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B Lymphocyte Development in Galt

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AID	Activation-induced cytidine deaminase
Ad	Adenovirus
APRIL	A proliferation-inducing ligand
Apx	Appendix
BCMA	B cell maturation antigen
BGG	Bovine gamma globulin
BM	Bone marrow
BCR	B cell receptor
BBR	BAFF-binding receptor
BRECs	B cell recombination excision circles
BR3	BAFF receptor 3
CHO	Chinese hamster ovary cells
CsA	Cyclosporine
CVF	Cobra venom factor
CV	Conventional
CVID	Common variable immunodeficiency
CLPs	Common lymphoid progenitors
D	Domes

DCs	Dendritic cells
DL1	Delta-like 1
F	Follicles
FAE	Follicle-associated epithelium
FL	Full length
GF	Germ free
GALT	Gut-associated lymphoid tissue
GCs	Germinal centers
HSA	Heat stable antigen
HSCT	Hematopoietic stem cell transplant
Ig	Immunoglobulin
MLN	Mesenteric lymph node
MZ	Marginal zone
PB	Peripheral blood
PP	Peyer's patch
SHM	Somatic hypermutation
SR	Sacculus rotundus
TLRs	Toll-like receptors
TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
VE	Villous epithelium
Xid	X-linked agammaglobulinemia

ABSTRACT

B lymphocytes constitute an important arm of the humoral immune system and protect us by generating antibodies against the foreign antigens we encounter every day. Mammals use different strategies, to develop and maintain the B cell arm of humoral immunity. In mice and humans, B cells develop in the bone marrow (BM), where the primary antibody repertoire is generated. The peripheral B cell compartment in the adults of these species is maintained by the continuous production of new B cells in the BM. In other species such as, rabbits, however, B cells develop differently. B cells that develop in the BM early in ontogeny, migrate to GALT, where in the presence of commensal bacteria, they undergo proliferative expansion and somatically diversify the immunoglobulin (Ig) genes. The B cells with a diversified repertoire then enter the circulation and serve as the pre-immune antibody repertoire. The mechanism(s) that regulate B cell expansion and diversification in GALT is not known. Further, unlike in mice, B lymphopoiesis in rabbits is not continuous. After about 3-4 months of age, B lymphopoiesis arrests, and it is unclear how the peripheral B cell compartment is maintained when there is no influx of newly-made B cells from the BM.

For my dissertation, I investigated how B cells develop in the GALT of rabbits, and how they are maintained in adults after the arrest of lymphopoiesis. To identify cellular signals that promote B cell expansion in GALT, I injected newborn rabbits with recombinant adenoviruses (rAd) that express soluble decoy receptors. Using this

approach, I identified several cell-cell and cell-cytokine interactions that are required for B cell development in GALT. I demonstrated that B cells in GALT expand in a CD40-CD40L, CD21-CD21L dependent, and B7-CD28 independent manner. By neutralizing a cytokine, B cell activating factor (BAFF), I demonstrated that BAFF is required for the differentiation of BM-derived immature B cells (also known as transitional B cells) into follicular and mature B cells in GALT and peripheral tissues, respectively. Further, I demonstrated that BAFF provides a survival signal to follicular B cells in GALT.

Using several cross-reactive antibodies, I identified subpopulations of transitional and mature B cells in young and adult rabbits. Transitional B cells, are the precursors to mature B cells, and are constantly replenished by the BM in mice and other species. As described in mice, I identified two populations of transitional B cells: T1 and T2 B cells in rabbits. Surprisingly, I found that these transitional B cells were present in GALT and peripheral tissues of adult rabbits, even in the absence of B lymphopoiesis. Unlike murine T1 B cells that are not proliferating *in vivo,* and have undiversified Ig genes, T1 B cells in rabbit were proliferating and were somatically diversified. The presence of transitional B cells in adults, long after the arrest of lymphopoiesis suggests that they are somehow maintained since their development early in ontogeny. Further, the presence of a diversified repertoire indicates that they have gone through a germinal center-like reaction and are not recent emigrants from the BM. I designate the somatically diversified transitional B cells in adults as T1d B cells and propose that they are maintained in GALT by self-renewal. I suggest that self-renewing T1d B cells continuously

differentiate into mature B cells, and thus maintain peripheral B cell homeostasis in adults, after the arrest of lymphopoiesis.

While investigating the role of BAFF during B cell development, I was surprised to find that most primary B cells in rabbit did not bind to recombinant soluble BAFF (rBAFF), even though they expressed BAFF receptor 3. I was surprised because in other species, essentially all B cells bind to rBAFF. I demonstrated that primary rabbit B cells do not bind to rBAFF, because the BAFF-binding receptors (BBRs) on B cells are occupied by endogenous soluble BAFF. I propose that the chronic occupancy of BBRs on primary B cells with endogenous BAFF, provides B cells with a tonic/survival signal, and consequently allows them to remain long-lived.

Taken together, my work provides insights into how B cells develop, and are maintained in adult rabbits. My work suggests that B cells in GALT develop in a T cell independent and BAFF and complement dependent manner. In adults, the peripheral B cell compartment is likely maintained by self-renewing T1d B cells and long-lived mature B cells.

CHAPTER ONE

LITERATURE REVIEW

Introduction

B lymphocytes constitute an important arm of the humoral immune system and protect us by generating antibodies against the foreign agents we encounter every day. Most of our knowledge about the biology of B lymphocytes is derived from studies using animal models. Historically, rabbits were used during the early days of immunology by several investigators to delineate some of the immunological concepts we know today, such as, allotypes (Oudin, 1956a, b) and allelic exclusion (Pernis et al., 1965, Cebra et al., 1966). In fact, some of the early studies to elucidate the structure of immunoglobulin (Ig) were performed using rabbits (Fleischman et al., 1963). Today, with the availability of several antibody reagents, transgenic and gene knock-out models, the mouse has become a popular choice for research in B cell immunology. Consequently, B cell development in mice is relatively well understood when compared to other species, including humans.

Since the early 70s, the focus of B cell development was to understand how the immunoglobulin genes were rearranged to generate a functional antibody molecule and to delineate the stages through which hematopoietic stem cells (HSCs) in the bone marrow (BM) progress to become a B cell (Welner et al., 2008). We now understand that HSCs

progress broadly through a proB and preB cell stage, during which the V, D and J genes rearrange before giving rise to an immature B cell that exits the BM and enters the periphery. Once in the periphery, BM-derived immature B cells, designated transitional B cells go through a series of developmental stages before becoming mature B cells (Carsetti et al., 1995, Loder et al., 1999). Several questions; however, remain about the fate of these transitional B cells once they enter the circulation. Only a fraction of the BM-derived transitional B cells enter the mature B cell repertoire (Allman et al., 1993, Melchers et al., 1995) and the pathways they use to develop into different mature B cell subsets are not completely understood (Thomas et al., 2006). Interest in elucidating this peripheral phase of B cell development was further prompted following the identification of a cytokine, B cell activating factor (BAFF) (Schneider et al., 1999) and its receptors (Gross et al., 2000, Thompson et al., 2001). Studies using BAFF and BAFF-receptor knock-out mice revealed that BAFF was required for the peripheral phase of B cell development from the transitional stage onwards (Schiemann et al., 2001, Thompson et al., 2001). Most of these and other studies to understand the peripheral phase of B cell development are performed in mice and it is unclear how many of these findings relate to those in other species. In humans, although many aspects of B cell development in the BM are known and appear similar to mice, very little is known about the peripheral stages of B cell development (Carsetti et al., 2004). As stated by scientists whom I would like to call the founding fathers of the field of comparative immunology, Robert Good and his protégé Max Cooper, the immune system of several species needs to be investigated to obtain a holistic understanding of the immune system (Flajnik, 2002).

In this chapter, I will review the current state of knowledge of B cell development in rabbits and compare that with what is known in other species. I will also raise some fundamental questions to be addressed, not only in rabbits, but that would also be applicable towards understanding B cell development in other species as well. In the latter part of this chapter, I will review the stages of peripheral B cell development, and the biology of transitional and mature B cell subsets as described in mouse and man.

Development of the primary antibody repertoire

Historically, perhaps one of the most fundamental and debated topics in immunology was the development of a primary antibody repertoire. How could our immune system generate antibodies that have an unlimited number of specificities? Susuma Tonegawa, first solved this problem and showed that in mice, a large amount of the primary antibody repertoire is generated by the random joining of multiple V, D and J gene segments during B cell development in the BM (Hozumi and Tonegawa, 1976, Tonegawa, 1988). After years of debate, this explanation seemed so complete that an additional or alternate strategy for generation of diversity was not envisioned. Further support for the idea that the antibody repertoire was generated by random joining came from studies in humans showing that the human Ig loci are structured and somatically rearranged in a manner identical to that in mouse (Alt et al., 1987). However, it soon became clear that "random joining" was not the only mechanism to generate antibody diversity. While investigating the mechanism for generating the primary antibody repertoire in other species, several investigators discovered that the random joining of Ig

genes contributed relatively little to the primary repertoire, and that they used different mechanisms: Gene conversion - a process by which portions of rearranged V genes are replaced by sequences donated from a multitude of upstream pseudo-Vgenes. Also, it was shown that somatic hypermutation (SHM), introduction of point mutations in rearranged Ig genes, contributed to the generation of antibody diversity.

In rabbits, the germline contains multiple V_H gene segments; however, only one of them, V_H1 is utilized in most B lymphocytes (Knight and Becker, 1990). Similarly, only two D_H gene segments (D2a and D2b) and one J_H gene segment (JH4) is preferentially utilized and expressed (Friedman et al., 1994). While the significance of such selective utilization of germline Ig genes is not known, it is clear that the B cells exiting the BM and bearing these rearranged VDJ genes will have only a limited specificity/repertoire. After birth, this repertoire is greatly expanded in the periphery through gene conversion and somatic hypermutation (Knight and Barrington, 1998). By 6-8 weeks of age, essentially all peripheral B cells have a diversified repertoire (Crane et al., 1996), and consequently, the rabbit is considered fully immunocompetent.

Similar to rabbits, chicken B cells also utilize only one V_H and one V_L gene segment in their V(D)J gene rearrangements (Reynaud et al., 1985, Reynaud et al., 1987) and the primary antibody repertoire is generated by the process of somatic gene conversion (Thompson and Neiman, 1987, Reynaud et al., 1989). However, unlike rabbits, the antibody repertoire is generated during embryogenesis, and consequently, chicks are born (hatched) with a full complement of diversified B cells (Reynaud et al.,

1994). Studies in other species such as, sheep, pigs and cattle, similarly provided evidence for selective/limited utilization of germline V genes during ontogeny and expansion of an initial limited repertoire by somatic hypermutation (Reynaud et al., 1991, Reynaud et al., 1995, Parng et al., 1996, Aitken et al., 1999, Butler et al., 2000).

Although, the random joining of V, D, and J genes in the BM is the predominant pathway of development of the primary antibody repertoire in mice and humans, there is some evidence that certain B cell subsets in these species generate their primary antibody repertoire using alternative strategies. Golby et al. (2002) described a unique subset of B cells in the human fetal intestine. These B cells had large cytoplasmic processes and were proliferating, despite the absence of exogenous (bacterial) antigen. They were $CD20^{+}IgM^{+}IgD^{+}$ Light $chain^{+}$ and were located beneath the epithelium and scattered through the lamina propria. Interestingly, these B cells were present only in fetal intestine, but not in the postnatal intestinal lamina propria. Analysis of V_H gene sequences from the fetal intestine revealed that some of these B cells had low levels of SHM. Does this B cell population develop in a pathway analogous to the "GALT" species? It will be interesting to monitor the fate of these B cells in postnatal life, after the intestinal tract is colonized by commensal microbiota. Will these B cells migrate to other sites and/or alter their morphology/phenotype and reside in the intestine to form GC-like structures?

Several lines of evidence suggest that IgM^{+} $IgD^{+}CD27^{+}$ B cells in humans develop by adopting some of the strategies seen in "GALT" species. Conventionally,

antibody diversity in humans is first generated by rearrangement of Ig genes during development in the BM, and later by T cell- and antigen-driven diversification in the germinal centers (GCs). We acquire the ability to respond to T-dependent antigens, form GCs and memory B cells soon after birth, but the ability to respond to T-independent antigens takes a few years to develop (Bauer et al., 2002, Zandvoort and Timens, 2002). Marginal zone (MZ) B cells are known to mediate an immune response against T-independent antigens. By performing gene-expression profiling, Weller et al. (2004) demonstrated that circulating $IgM^{+}IgD^{+}CD27^{+}$ B cells are MZ B cells and by analyzing this subset in children below 2 years of age; they found that the V_H genes from these B cells exhibited SHM. By performing spectratype analysis, they found no evidence of clonal selection; even though the children in the study were vaccinated, and thus exposed to T-dependent antigens (Weller et al., 2008). The presence of $IgM^{+}IgD^{+}CD27^{+}$ B cells with mutated Ig genes in very young children suggests that these B cells are developmentally programmed to diversify their repertoire during ontogeny. Further, the presence of this B cell population in patients who are unable to form GCs due to a mutation in CD40L gene suggests that these B cells can develop outside the boundaries of a classic T-B cell interaction, which is a hallmark of GC reaction (Weller et al., 2001). These findings are reminiscent of sheep B cells that diversity their repertoire by SHM and develop in an antigen and T cell independent manner (Reynaud et al., 1991, Reynaud et al., 1995). I predict that upon closer observation, human B cell subsets that utilize somatic gene-conversion will also be discovered. While investigating the mechanism of isotype switching in an IgH transgenic mice, Gerstein et al. (1990) demonstrated that the

VDJ transgenes exhibited a somatic gene conversion-like process. More recently, Shimomura et al. (2008) described a novel subset of B cells in the murine large intestine that diversified the Ig genes by SHM. These B cells developed in an antigen and T cell independent manner, and unlike conventional B cells, they were $CD21^-$ and $CD23^-$ (Shimomura et al., 2008).

Thus, the primary antibody repertoire in species can be developed through more than one strategy, even though one mechanism appears to predominate. Rabbits, are unusual among mammals in that they utilize both SHM and gene conversion to diversify their repertoire (Knight and Barrington, 1998). I submit that alternate strategies to generate and express a primary BCR repertoire are not likely to have been discovered if studies had focused only on B cells from mouse and human.

Role of GALT in B cell development

When the chicken model of B cell development was first described, it not only indicated that an alternate strategy for generation of the antibody diversity exists, but also it introduced gut-associated lymphoid tissues (GALT) as a site where the rearranged V(D)J genes diversified (Weill and Reynaud, 1987). The bursa of Fabricius, which is part of GALT, is a primary organ for B cell development in chickens. During ontogeny, embryonic stem cells colonize the bursa and differentiate into B lymphocytes. These B cells then undergo a proliferative expansion, form organized follicles, and somatically diversify the Ig genes. The diversified B cells then leave the bursa and constitute the peripheral B cell compartment. By 6 months of age, the bursa involutes completely, but

the B cells generated early in ontogeny are maintained throughout the life of the bird (Pink, 1986, Pink and Lassila, 1987).

After the initial description of the role of bursa of Fabricius in chicken (Glick, 1956, Mueller et al., 1959), many investigators hunted for the mammalian homologue. The rabbit appendix received much attention then and was proposed to be the bursal-equivalent (Archer et al., 1963, Cooper et al., 1966). This hypothesis was supported by studies that demonstrated that rabbits appendectomized at birth had significantly reduced serum Ig levels and reduced antigen-specific Ig responses (Sutherland et al., 1964, Archer et al., 1964, Cooper et al., 1968). These observations were similar to those in chicken; bursectomy in the embryo resulted in severe agammaglobulinemia (Glick, 1956, Mueller et al., 1959, Cooper et al., 1965). Subsequently, the ileal Peyer's patch (PP) of sheep and ruminants were also proposed to be a bursal-equivalent (Reynolds and Morris, 1983, Landsverk, 1984).

Now it is well established that GALT plays an important role in B cell development in several species. In sheep, clusters of IgM^+ B cells are first detected in the spleen at about day 48 of gestation (the gestation period in sheep is 150 days). Neither the origin of these B cells nor the stages of development prior to day 48 is known. After expanding in number in the spleen, the IgM^+ B cells seed the ileal PP at about day 68. Here B cells proliferate vigorously, form organized follicles, and somatically diversify the Ig genes. About 5% of these diversified B cells leave the PP and enter the circulation, while the remaining B cells die by apoptosis (Reynolds, 1997). The importance of ileal

PP in B cell development was demonstrated by its surgical removal; excision of ileal PP from neonates caused a severe reduction in the number of peripheral B cells (Gerber et al., 1986). These results again are reminiscent of similar studies of the chicken bursa. Similar to the bursa, the ileal PP involutes with age (by about 15 months of age), and the B cells generated early in ontogeny are maintained throughout life (Reynolds, 1997).

In rabbits, B lineage cells are detected in fetal liver (Tunyaplin and Knight, 1995), and in other sites, such as fetal omentum and spleen (Solvason and Kearney, 1992, McElroy et al., 1981). Late in fetal life, B cell development switches from liver to the BM. After birth, BM B cells that have rearranged VDJ genes exit the BM to seed the peripheral tissues. These B cells migrate to GALT, where they undergo proliferative expansion, form follicular structures, and somatically diversity the Ig genes. As in other species described above, the diversified B cells from GALT seed the peripheral tissues and constitute the primary B cell compartment (Knight and Winstead, 1997). Vajdy et al. (1998) directly demonstrated the role of GALT in B cell development; surgical excision of all organized GALT at birth led to the generation of undiversified or poorly diversified B cells in the periphery. Additionally, the authors found a decline in the frequency of peripheral B cells after removal of the GALT. These findings not only indicate that GALT is the site for generation of diversity, but also indicates that GALT functions as a primary lymphoid organ for the post-natal expansion of B cells. Unlike, chicken bursa and sheep ileal PP, the rabbit appendix does not involute with age. Instead, the appendix undergoes a few morphological changes and is thought to function as a secondary

lymphoid organ in adult rabbits (Weinstein et al., 1994b). Another notable distinction is that, unlike in chicken and sheep that develop their antibody repertoire during fetal life, independent of exogenous (bacterial) antigen, B cell development in rabbit requires antigen (Lanning et al., 2000b). Somatic diversification of the Ig genes begins only after birth, and after the GALT is colonized by commensal bacteria. Using a germ-free appendix model, Rhee et al. (2004) demonstrated that both proliferative expansion of B cells and somatic diversification of VDJ genes in the appendix are dependent on the presence of commensal bacteria. To investigate the mechanism by which bacteria promote B cell development in GALT, Severson et al. (2010) tested whether bacterial-derived superantigen-like molecules could mediate B cell proliferation in GALT. Superantigens are molecules that bind outside the conventional antigen binding site and because almost all B cells in rabbit utilize the same V_H gene (V_H1) during VDJ gene rearrangements, a superantigen could readily interact with all B cells, independent of their BCR specificity (Knight and Winstead, 1997). In support of this idea, Severson et al. (2010) identified and described a superantigen-like protein, ExsK, found on the surface of bacterial spores. They demonstrated that surface proteins on bacterial spores promote B cell development in GALT (Severson et al., 2010).

Much less is known of the mechanism of B cell development in larger animals such as swine and cattle. B cells in pigs essentially appear to follow a similar developmental pattern as in rabbits, even though there are some differences in B cell development at the molecular level (Sun and Butler, 1996, Sun et al., 1998, Butler et al.,

1996). In pigs, liver is the primary site for B cell development in fetal life until the BM takes over (Sinkora et al., 2002). Thereafter B cells with rearranged VDJ genes diversify the Ig genes and studies with germ-free piglets revealed that this process of diversification after birth requires bacteria (Butler et al., 2000). However, there is no evidence that swine use GALT as a site for repertoire diversification. The ileal PP are thought to be a part of the mucosal immune system, rather than a primary lymphoid tissue during ontogeny (Sinkora et al., 2002). Ig gene diversification in cattle occurs during fetal development and does not require intestinal microbiota (Lucier et al., 1998). Similar to sheep, the ileal PP in cattle is a site for generation of the antibody repertoire. In both these species, although exogenous antigen is not required for development of the antibody repertoire, it is required for the maintenance of B cells in the follicles (Yasuda et al., 2002, Yasuda et al., 2006). In summary, B cells in chickens, sheep and cattle diversify the antibody repertoire during fetal life and independent of exogenous bacterial antigens. In contrast B cells in rabbits and pigs diversify the antibody repertoire after birth and require antigen.

Is there a role for GALT during B cell development in mice? During the time that immunologists were searching for a bursal-equivalent in mammals, the role of murine PP was also examined. Friedberg and Weissman (1974) hypothesized that if PP functioned like a bursal-equivalent, then B cells will be exclusively produced in the PP of neonatal mouse. They monitored the rate of accumulation of B cells in different tissues of newborn mice and found that the spleen had a greater rate of accumulation of B cells

compared to PP and lymph nodes. They concluded that the modest levels of proliferation in the PP could not account for the rise in circulating B cells in neonatal mice and suggested that the spleen rather than PP might serve as a primary lymphoid organ. Subsequent studies using [^{3}H]-thymidine labeling and adoptive transfer experiments revealed that the BM contained B lineage precursors and was the source for B cell production in postnatal mice (Osmond and Nossal, 1974a, b, Stocker et al., 1974, Owen et al., 1977, Andrew and Owen, 1978). Around that time, PPs were found to be the source of precursors for IgA plasma cells (Craig and Cebra, 1971) and studies investigating the role of IgA in the gut gave rise to the notion that PPs were responsible for local humoral immunity rather than as sites for B cell production (Cebra et al., 1977).

The contribution of ileal PP in sheep and appendix in rabbit could be directly examined by their surgical removal (Gerber et al., 1986, Vajdy et al., 1998). However, because comparable experiments in fetal or neonatal mice cannot be performed due to technical limitations, I think a role for PPs or other murine GALT during B cell development cannot be ruled out. Although GALT is not a site for generation of the antibody repertoire in mice, it may play a role in the development of some B cell subsets (Shimomura et al., 2008). Similarly, in humans, although BM appears to the predominant pathway for B cell development, I think some aspects of the GALT pathway may be conserved. What could be the function of the 200-300 PPs (Cornes, 1965) located along the human intestinal tract? Is there any role for the human appendix in B cell development? Rabbit B cells go through human and murine-like developmental stages in

the BM (see next section) and a chicken and sheep-like development in the GALT. Thus, rabbits serve as a unique model to investigate the biology of B lymphocytes.

B lymphopoiesis in the BM

B lymphopoiesis, which is defined as the maturation of HSCs to naïve B cells, is mediated through several stages and occurs predominantly in the BM of mammals. When considering B cell development in sheep and chicken, much of the emphasis, however, is on the ileal PP and bursa, respectively. One organ that is conspicuous by its apparent lack of importance in these species is the BM. Cytological and flow cytometric analysis in these species revealed that the BM contains few, if any, B lineage cells when compared to rodents (Reynolds, 1997). HSCs in rodents go through proB and preB cell stages before giving rise to B cells in the BM. The ordered rearrangement of Ig genes begins in proB cells where D_H to J_H gene rearrangements occur. These cells progress to preB cells following rearrangement of the V to DJ gene and express a preB cell receptor (preBCR). Signaling through the preBCR is required for the developmental progression into the next stage, an immature B cell (Kitamura et al., 1992). In sheep, there is no evidence for ProB and preB cells and essentially nothing is known about the early stages of B cell development (Reynolds, 1997). In contrast, in rabbits, B-lineage precursors and the stages of B cell development in the BM are relatively well described. Several groups reported the presence of preB cells in different tissue during fetal and neonatal life (Hayward et al., 1978, McElroy et al., 1981, Solvason and Kearney, 1992, Jasper et al., 2003). These preB cells first appear in fetal BM at day 25 of gestation, and increase in

number after birth (Hayward et al., 1978, McElroy et al., 1981, Gathings et al., 1981, Gathings et al., 1982). Jasper et al. (2003) described proB cells in rabbits and followed the kinetics of appearance of both proB and preB cells after birth. They found that the percentage of these precursors in the BM peaked at ~ 2-3 weeks of age and reported that approximately 70% of the B-lineage cells were proB cells and the remaining 30% of the cells were made up of preB and B cells. Additionally, Jasper et al. (2003) provided evidence for the expression and formation of a pre-BCR-like complex during B cell development in the BM. Taken together, these studies suggest that early B cell development in rabbits proceeds in a fashion similar to that in mice and humans.

B lymphopoiesis in mice and humans continues throughout life. Several lines of evidence indicate that B lymphopoiesis in rabbits, however, does not continue throughout life. The earliest evidence came in the form of allotype suppression experiments. Following administration of anti-allotype antibodies to neonatal rabbits, the targeted allotype could not be detected in the serum of injected rabbits for up to two years (Mage and Dray, 1965), presumably due to depletion of the targeted allotype bearing B cells. If B lymphopoiesis was ongoing, then one would expect to find new B cells, and consequently re-detect the suppressed allotype in the serum. This was the case when comparable experiments were performed in mice. In IgH-suppressed mice, new B cells of the suppressed allotype appeared within 6 weeks of birth (Lalor et al., 1989).

Years later, Crane et al. (1996) examined B lymphopoiesis in rabbits at the molecular level. The authors hypothesized that if B lymphopoiesis in the BM was

ongoing in adult life, then one would expect to find: a) B cells with undiversified VDJ genes, and b) progenitor B cells undergoing VDJ recombination. Using an RNase protection assay with a germ-line V_H gene (V_H1) as a probe, the authors did not find any evidence for the presence of undiversified VDJ genes in the BM of adult rabbits. By PCR-amplifying B cell recombination excision circles (BRECs), products of VDJ gene recombination events, the authors found highly reduced levels of BRECs in the BM of adult rabbits relative to the levels found in the BM of newborn rabbits. These studies indicated that B lymphopoiesis does not continue through adulthood in rabbits.

Jasper et al. (2003) followed the kinetics of appearance of B lineage precursors in the BM and provided evidence at the cellular level for the decline in B lymphopoiesis with age. As mentioned before, these authors found that the frequency of proB and preB cells peaked during the first 2-3 weeks of age. However, after this time period, the frequency of these cells declined gradually. By 16 weeks of age, no proB or preB cells were detectable in the BM by flow cytometry. The authors also quantified this decline in B lymphopoiesis at the molecular level. They measured the levels of BRECs in the BM from different aged rabbits and estimated that by 16 weeks of age, B lymphopoiesis in rabbits declines by over 99% when compared to newborn rabbits. Taken together, these data confirmed previous findings by Crane et al and established the kinetics of the decline in B lymphopoiesis.

B lymphopoiesis in the mouse, although continuous throughout life, also declines with age. Using Brdu labeling experiments, Labrie et al. (2004) demonstrated that the

number and production rate of preB cells are reduced ~4 fold in adult mice, when compared with young animals. Similarly, proB cells and common lymphoid progenitors (CLPs) have also been reported to decline in frequency with age (Min et al., 2006). What could be the reason for this decline in B lymphopoiesis? Are there intrinsic defects in the differentiation capacity of aged precursors, and/or changes in the BM microenvironment with age? By performing reciprocal adoptive-transfer experiments, Lebrie et al. (2004) demonstrated that when B cell precursors from both young and adult donor bone marrow were transferred into young irradiated recipients, B cell subpopulations of identical magnitude, turnover, and renewal rates were generated. In contrast, when transferred to aged recipients, B cell precursors from bone marrow of a young mouse gave rise to relatively fewer B cells. Notably, the pre-B cell compartment that was generated after the transfer was similar to that found in aged mice, with respect to production and turnover rates (Labrie et al., 2004). These studies suggest that age-related changes in the BM environment, rather than defects in the differentiation potential of B lineage precursors underlie the reduced output of B cells by the BM. While investigating the effects of aging on early B lineage precursors in the BM, Min et al. (2006) reported that both CLPs and pro-B cells from aged mice undergo reduced levels of homeostatic proliferation when compared to their respective counterparts in young animals. Because CLPs from aged mice exhibited a reduced capacity to give rise to its progenies, the authors suggested that aging might also influence the differentiation capabilities of B lineage progenitors (Min et al., 2006).

To investigate the mechanism(s) by which B lymphopoiesis arrests in rabbits, Kalis et al. (2007) first determined if the adult BM had any lymphopoietic potential. The authors transferred BM from adult rabbits into young rabbits and found donor-derived preB cells and T lineage precursor cells arising in the recipients. Consequently, they concluded that the adult BM contained a lymphoid progenitor (LP)-like stage that can give rise to both B and T cells and that B lymphopoiesis arrests at this early LP-like stage. This conclusion was further supported by the finding that B lymphopoiesis in adult rabbits could be re-initiated after sub-lethal irradiation. Using anti-MHC II mAb and recombinant IL7 as reagents, the authors identified and functionally characterized a population of $MHCII^{-}IL7$-binding cells that contained putative early lymphoid progenitor (ELP)-like cells. Preliminary investigations in the Knight lab using antibodies to CD24, c-kit and CD79a revealed that the $MHCII^{-}$ IL7-binding population contains a CLP-like population, that resemble murine CLPs both phenotypically and functionally (B.S., V.Y., and K.L.K., unpublished observations). It remains to be determined if the frequency and the differentiation potential of these CLP-like cells are altered between young and adult rabbits.

In humans, although little is known about age-related changes in B lineage precursors in the BM, there is considerable information regarding changes in peripheral B cell subsets with age. Several reports indicate that the total number of $CD19^{+}$ B cells decrease with age (Paganelli et al., 1992, Olsson et al., 2000, Breitbart et al., 2002, Chong et al., 2005, Frasca et al., 2008), presumably due to a decline in B lymphopoiesis. One of

the consequences of a decline in B cell immunity is a poor response to vaccinations. Understanding the mechanism(s) by which B lymphopoiesis declines with age will be useful in identifying therapeutic interventions to restore and maintain B cell immunity in the elderly.

Peripheral B cell homeostasis

Although B lymphopoiesis in rabbits arrests a few months after birth, rabbits are able to maintain robust B cell immunity throughout life. In fact, adult rabbits are widely used to generate high affinity antibody reagents for use in clinical and research laboratories. How is the B cell compartment maintained in adult rabbits? Perhaps, rabbit B cells are long-lived and/or self-renewing (Knight and Winstead, 1997). This idea is supported by the finding that adoptively transferred B cells can be detected for up to 20 months in the recipient rabbit (Adler et al., 1983). This number is considerably higher when compared to the life-span of murine B cells. Although variable results were obtained, presumably due of the different approaches used (such as [^{3}H] and Brdu labeling, adoptive transfers and administration of chemical drugs), the life-span of murine B cells is estimated to range from only a few weeks (~4-6) to months (~4-5) (Fulcher and Basten, 1997, Hao and Rajewsky, 2001). In mice, B1 B cells are maintained by self-renewal, unlike conventional B 2 B cells that are continuously replenished (Hao and Rajewsky, 2001). Although murine-like B1 B cells have not been identified in other species, it is possible that rabbit B cells functionally resemble B1 B cells and self-renew.

Even in species that exhibit continuous lymphopoiesis, the mechanisms that regulate the size and composition of the peripheral B cell compartment are not completely understood. Even though lymphopoiesis is ongoing and a large number of B cells are generated in the BM, only a fraction of these newly-formed cells (~3%) successfully enter the B cell pool (Carsetti et al., 1995). Following experimentally-induced ablation of B cell development in the BM of adult mice, peripheral B cells still persist. However, their composition is different; only B1 and MZ B cells remain while virtually no B2 B cells are found (Hao and Rajewsky, 2001). These observations indicate that the biology of MZ B cells is different from that of conventional follicular B cells. Are MZ B cells long-lived? Do they also self-renew like B1 cells? Because B lymphopoiesis in rabbits arrests so early in life, the humoral immune system must have developed strategies to maintain B cell immunity throughout life. Investigating these strategies will provide insights into how B cells are maintained not only in rabbits, but also, likely, in other species.

Transitional B cells: Intermediates during peripheral stages of B cell maturation

During B cell development, the BM-derived immature B cells, designated transitional B cells, exit the BM and migrate to the peripheral tissues where they develop into mature B cells (Carsetti et al., 1995, Loder et al., 1999). These transitional B cells are identified by several cell surface markers expressed on newly formed B cells in the BM. In mice, these cells were first identified based on the high expression of heat stable antigen (HSA), a murine homologue of human CD24; these cells were distinguished from

mature B cells that were HSA^{lo} (Allman et al., 1992). HSA^{hi} B cells in the periphery differ from HSA^{lo} B cells in several aspects. First, HSA^{hi} B cells arise before HSA^{lo} B cells both during ontogeny and during reconstitution of irradiated adults. Second, HSA^{hi} B cells in the periphery turnover with 4 days, while HSA^{lo} B cells are long-lived. Third, HSA^{hi} B cells fail to enter cell cycle following BCR crosslinking, while HSA^{lo} B cells readily enter cell cycle under similar conditions (Allman et al., 1992, Allman et al., 1993). More recently, the HSA^{hi} cells are referred to as transitional B cells.

Subsequently, Loder et al. (1999) and Allman et al. (2001) demonstrated that transitional B cells in the periphery could be divided into distinct subsets based on surface phenotypes. Based on the differential expression of CD21, CD23 and IgD, Loder et al. (1999) classified the HSA^{hi} transitional B cells into two stages: transitional type 1 (T1) and type 2 (T2). They found T1 B cells in the BM, blood and spleen, while T2 B cells were found exclusively in the spleen. To investigate the developmental relationship between T1, T2, and mature B cells, the authors performed adoptive transfers. They transferred purified T1 B cells into RAG-2 deficient mice, which lack B and T cells, and detected T2 and mature B cells in the periphery of the recipient mice. Further, following adoptive transfer of T2 B cells, they detected only mature B cells in the recipients. These findings indicated that T1 B cells were the precursors of T2 and mature B cells and that T2 B cells develop into mature B cells. Further, using mice with genetic deletion of CD45 and Igα, molecules required for BCR signaling, the authors showed that the development of T2 and mature B cells from T1 cells is dependent on defined signals

derived from the BCR. Based on these findings, they proposed a linear T1→T2→M pathway for peripheral B cell maturation (Loder et al., 1999). Using a C1q receptor homologue, AA4, Allman et al. (2001) identified 3 populations of transitional B cells based on the surface expression of CD23 and IgM: $AA4^{+}CD23^{-}IgM^{hi}$ (T1), $AA4^{+}CD23^{+}IgM^{hi}$ (T2), and $AA4^{+}CD23^{-}IgM^{lo}$ (T3). They confirmed that these subsets are functionally immature, based on their high turnover rates and inability to proliferate following BCR crosslinking. They also demonstrated that the T1 B cells in their scheme of classification are the earliest precursors to subsequent stages of B cells during development.

One major discrepancy between the two schemes of classification described above is the nature of the late stage transitional B cells. Carstetti and colleagues found that ~ 20% of the T2 B cells were proliferating *in vivo*. Consequently, they proposed that the late stages of B cell maturation are associated with a proliferative burst that may serve to increase the frequency and number of useful clones entering the mature B cell pool (Loder et al., 1999, Srivastava et al., 2005). In contrast, none of the three stages of transitional B cells in the Allman et al. (2001) scheme of classification were proliferating *in vivo*. By performing multi-color flow cytometric analysis, they found that T2 B cells in the Loder et al. scheme did not correspond to their $AA4^{+}$ B cell subsets. Because HSA is also expressed at high levels on MZ B cells, they suggested that the Carstetti group's T2 B cell gate may include some proliferating MZ B cells (Srivastava et al., 2005). Thus, both these models differ in the characterization of the late stage T2 (and T3) B cells.

Even though the strategies used to subdivide transitional stages vary, these findings nevertheless indicate that BM-immature→transitional→mature is likely the major route of maturation for primary B cells.

Less is known about transitional B cells and the peripheral stages of B cell development in humans. Carsetti et al.(2004) first attempted to identify transitional B cells in the blood using a combination of markers that recognized mature naïve, and memory B cells, and also B lineage precursors in the BM. Accordingly, they used anti-CD27 to first eliminate mature memory B cells from their analysis, and identified a single population of transitional B cells that was $CD24^{hi}CD38^{hi}$ $CD10^{+}$. Subsequently, other investigators identified transitional B cells and classified them as T1-like and T2-like based on the differential expression of CD24 and CD38 (Marie-Cardine et al., 2008) or IgD and CD38 (Sims et al., 2005). Recently, Suryani et al. (2010), using CD21 as a marker, identified two transitional B cell subsets ($CD21^{lo}$ and $CD21^{hi}$) and demonstrated that the $CD21^{lo}$ subset is the precursor to the $CD21^{hi}$ B cells. Functionally, human transitional B cells are similar to murine transitional B cells in that they have largely unmutated Ig genes and do not proliferate upon BCR crosslinking (Sims et al., 2005).

The study of B cell subpopulations in individuals with primary B cell deficiencies and in patients undergoing hematopoietic stem cell transplants (HSCTs) provided an opportunity to understand the biology of transitional B cells in humans. Patients with X-linked agammaglobulinemia (Xid) exhibit a block in B cell development in the BM due to impaired BCR signaling (Conley et al., 2000). Consequently, they have drastically

reduced numbers of B cells in the periphery. Suryani et al. (2010) analyzed the residual B cells in these patients and found that were predominantly $CD10^{+}$ and $CD21^{lo}$ transitional B cells. Further, when they analyzed the kinetics of B cell reconstitution in a cohort of patients undergoing HSCT, they found that the $CD21^{lo}CD10^{+}$ B cells were the first subset of B cells to appear (Suryani et al., 2010). These findings indicate that these transitional B cells are the precursors to mature B cells during development. Murine transitional B cells turnover rapidly and differentiate into mature B cells within 4 days and are replenished continually from the BM (Allman et al., 1993). In contrast, studies from B cell depleted and irradiated HSCT patients revealed that human transitional B cells are relatively long-lived. Transitional B cells normally constitute 2-5% of the B cells in adult peripheral blood (PB); however, after treatment with anti-CD20 (Rituximab™), nearly all of the reconstituted B cells in PB are transitional B cells ($CD24^{++}CD38^{++}CD10^{+}$) and these cells remain at this frequency for approximately one year (Palanichamy et al., 2009). Similarly, five months after HSCT, up to 50% of the B cells in PB are transitional B cells (Marie-Cardine et al., 2008). These data suggest that the turnover rate of transitional B cells in humans is likely much longer than in mice.

Research from the past decade or so has established transitional B cells as a crucial link between immature B cells in the BM and mature B cells in the periphery. While many studies of peripheral B cell development have been performed in mice, and to a certain extent in humans, essentially no such studies are available in rabbits or other species that utilize GALT for some aspects of B cell development (Weinstein et al.,

1994a, Reynaud et al., 1991, Meyer et al., 1997, Parng et al., 1996, Butler et al., 2000). In rabbits, and likely in other species, because B lymphopoiesis in primary lymphoid organs abates early in life (Crane et al., 1996, Jasper et al., 2003), it is unclear how the peripheral B cell compartment is maintained. Does B cell maturation progress through transitional-like B cell intermediates in these species? Do transitional-like B cells or other intermediates exist in these species? Because the chicken bursa, sheep ileal PP and rabbit appendix are important site for B cell development, the B cells emanating from these organs and trafficking to the peripheral tissues have been proposed to function as equivalents of transitional B cells in these species (Weill and Reynaud, 2005).

Role of BAFF and APRIL during peripheral B cell development

B cell Activating Factor (BAFF), discovered almost a decade ago, has emerged as an important regulator of peripheral B cell homeostasis and survival. Schneider et al. (1999) first identified and characterized human and murine BAFF and demonstrated that BAFF functioned as a co-stimulatory molecule to induce B cell proliferation *in vitro*. Further, using recombinant BAFF (rBAFF) as a tool, the authors discovered that rBAFF bound predominantly to B cells, suggesting that BAFF-receptor is expressed only on B cells (Schneider et al., 1999). Subsequent studies by other investigators revealed that BAFF bound to three receptors: BAFF-R/BR3 (BAFF receptor 3), TACI (Transmembrane activator and CAML interactor), and BCMA (B cell maturation antigen) (Gross et al., 2000, Thompson et al., 2000, Thompson et al., 2001). Around this time, another closely related cytokine APRIL (A Proliferation Inducing Ligand) was also

identified and was found to bind to two of these receptors: BCMA and TACI (Hahne et al., 1998, Wu et al., 2000, Yu et al., 2000).

Identification of these novel cytokines and their receptors led several investigators to develop gene knock-out and transgenic animal models to evaluate their function(s) *in vivo*. Mice deficient in BAFF or its receptor BR3 exhibited a block in B cell development beyond the transitional T1 stage and lacked marginal zone and conventional B2 B cells, demonstrating that BAFF is required for peripheral B cell maturation and survival (Thompson et al., 2001, Schiemann et al., 2001, Gross et al., 2001). In contrast, overexpression of BAFF resulted in an accumulation of B cells and development of an autoimmune-like phenotype, indicating that optimal levels of BAFF are required for peripheral B cell development (Mackay et al., 1999). Mice deficient in TACI had increased numbers of B2 B cells and serum Ig, suggesting that this BAFF/APRIL receptor has a negative regulatory role in controlling the size of the B cell pool (Yan et al., 2001). B cell maturation appears normal in both APRIL and BCMA deficient mice; however, class switching to IgA, and survival of long-lived BM plasma cells were impaired in *April*$^{-/-}$ and *BCMA*$^{-/-}$ mice, respectively (Varfolomeev et al., 2004, Castigli et al., 2004, O'Connor et al., 2004). These findings demonstrate that BAFF and APRIL have distinct roles in B cell biology.

In humans, BAFF and its receptors appear to have functions similar to those in mice. For example, CVID (Common Variable Immunodeficiency) patients that lack TACI have increased numbers of B cells, but unlike mice, have decreased Ig levels

(Salzer et al., 2005, Salzer et al., 2009), suggesting that in humans, TACI plays a role in regulating both the size of the B cell compartment and Ig production . Similar to BR3 deficient mice, CVID patients that lack BR3 also develop severe B cell lymphopenia due to the arrest of B cell development at the transitional B cell stage (Warnatz et al., 2009). Even though these phenotypes do not manifest until adulthood, these findings show that human B cells also rely on BAFF signals for their maturation and survival.

Since the discovery of BAFF in humans and mice, BAFF has been cloned from chickens, ducks, quails, pigs, and rabbits, indicating that BAFF is evolutionarily conserved (Schneider et al., 2004, Guan et al., 2007a, b, c, Chen et al., 2009). However, the receptors for BAFF do not appear to be conserved. In chickens, no evidence for homologues of TACI, BCMA and APRIL were found, leaving birds with only BAFF and a single receptor, BR3 to control their B cell compartment. In young chicks, *in vivo* neutralization of BAFF with a decoy receptor severely impaired B cell development in the bursa, demonstrating that BAFF is required for development of bursal B cells (Kothlow et al., 2007, Reddy et al., 2008).

Unlike mice, the role of BAFF in other mammals such as, rabbits, sheep, cattle and pigs is not known. Given the crucial role that BAFF plays in murine B cell homeostasis and survival, it is tempting to speculate that BAFF may play similar overlapping and/or unique roles in other mammals.

Mature B cell subsets: Phenotypes, location and functions

Mature B cells in mammals have been variously divided into subsets based on criteria such as surface phenotype, location and function. In mice, B cells (identified by a pan B cell marker, B220) are generally classified as B1 and B2 B cells (also known as conventional B cells). The B2 B cells constitute virtually the entire B cell compartment in the peripheral lymphoid organs. They are $CD23^{+}$ HSA^{lo} and express low levels of IgM, and high levels of IgD (Well et al., 1995). In the spleen, in addition to B2 B cells that occupy the follicular zone and the red pulp, a small number of B cells (5-10%) reside in the MZ, which is a major antigen filtering and scavenging area (Martin and Kearney, 2000). Due to their location, MZ B cells are among one of the first cell-types to respond to blood borne antigens and thus function as a first line of defense. Unlike conventional B2 B cells that dependent on T cell help in the GCs to differentiate into antibody secreting cells, MZ B cells can rapidly differentiate into plasmablasts without any T cell help. These features enable MZ B cells to mount a rapid immune response. Phenotypically, they are IgM^{hi}, IgD^{lo}, HSA^{hi} and $CD23^{-}$ (Wells et al., 1995). In rodents, they appear restricted to the spleen and are not circulating. Further, they have largely unmutated Ig genes (Makowska et al., 1999, Dammers et al., 2000, Allman and Pillai, 2008). In addition to MZ B cells, the spleen also contains a small number of B1 B cells that phenotypically and functionally resemble MZ B cells ($IgM^{hi}IgD^{lo}HSA^{hi}CD23^{-}$) and also express CD5 and CD43. These B1 B cells are typically found in the peritoneal and pleural cavities, where they represent the predominant B cell population. Two functions

have been attributed to B1 B cells: response to T independent antigens and production of natural antibodies (Hayakawa and Hardy, 2000, Martin and Kearney, 2001). In the peritoneal cavity, they contribute to the generation of IgM responses to T-independent antigens such as phosphorylcholine, an antigen present on many pathogenic bacteria. They also migrate to the mucosal sites and contribute to the generation of T-independent IgA responses (Allman and Pillai, 2008). One of the most distinctive features of B1 B cells is the presence of a restricted BCR repertoire to certain self and bacterial antigens. The antibodies they secrete are thought to aid in the clearance of common bacterial and viral pathogens (Ochsenbein et al., 1999, Baumgarth et al., 2000). Thus, similar to MZ B cells, B1 B cells also function as a first line of defense.

In addition to the unique phenotypic and anatomic localization, B1 and B2 B cells also differ in their developmental pathway. In adults, B2 B cells which account for ~95% of the B cells in the spleen and lymph nodes, are continually replenished by *de novo* production of B cells in the BM (Hayakawa et al., 1985, Hayakawa et al., 1986, Hao and Rajewsky, 2001). They can be readily reconstituted in irradiated recipients by transfer of undifferentiated BM cells, but not by transfer of mature conventional B cells (Wells et al., 1995). In contrast, B1 B cells develop only early in ontogeny and are maintained thereafter by self-renewal (Hayakawa et al., 1986, Hao and Rajewsky, 2001). They are reconstituted by transfers of fetal and neonatal sources of lymphoid precursors, but not by transfers of adult BM precursors, suggesting that the adult BM does not contain precursors for B1 B cells (Hayakawa et al., 1985, Solvason et al., 1991, Kantor et al.,

1992). However, recently, Esplin et al. (2009) identified a progenitor for B1 cells in the adult BM, suggesting that some B1 B cells can also be derived during adult life. Thus, the development of B1 and B2 lineage B cells in mice is an ongoing area of investigation.

In addition to B1, B2 and MZ B cells, another subset of mature B cells known as Bw B cells was recently described in mouse. Thiriot et al. (2007) analyzed several wild-derived outbred strains of mice along with the conventional laboratory raised inbred mice and identified a unique population of B cells, termed Bw B cells, that was conserved among the genus, *Mus*. The phenotype they exhibited ($CD5^{-}Mac\text{-}1^{+}B220^{hi}IgM^{hi}IgD^{hi}$ $CD43^{-}CD9^{-}$) were distinct from both B1 and B2 B cells and these cells were located in the spleen, peritoneal cavity and PB. By performing adoptive transfers of either fetal liver cells or T and B cell depleted BM cells from adult mice into Rag2 γc deficient mice; the authors detected the development of Bw cells, indicating that the precursors for Bw cells are present in both fetal and adult life. Functionally, Bw cells appeared to possess a restricted BCR repertoire and secreted natural antibodies like B1 B cells. Lastly, in addition to describing a novel subset of mature B cells, these authors also introduced wild-derived mice as another experimental model to study the development and function of B cell subsets (Thiriot et al., 2007).

In humans, mature B cell subsets are largely defined based on their developmental stages and functional characteristics such as, mutational status of Ig genes, isotype switching and ability to secrete antigen-specific antibodies. Accordingly, using a combination of surface IgD, CD38 and other markers, tonsil B cells were divided into

five subsets: "naïve" BM-derived B cells ($IgD^{+}CD38^{-}$) were separated based on the expression of CD23 into Bm1 ($CD23^{-}$) and Bm2 ($CD23^{+}$) subsets. Germinal center B cells ($IgD^{-}CD38^{+}$) were further delineated by CD77 into centrocytes (Bm3) and centroblasts (Bm4). The $IgD^{-}CD38^{-}$ cells were classified as memory B cells (Pascual et al., 1994, Liu and Arpin, 1997). With the identification of CD27 as a marker for memory B cells in humans (Agematsu et al., 2000), a simpler form of classification describing $CD27^{-}$ B cells as naïve and $CD27^{+}$ B cells as memory B cells emerged (Weill et al., 2009). Memory B cells localize in the splenic marginal zone (Liu et al., 1988, Tangye et al., 1998) and thus CD27 also serves as a marker for MZ B cells in humans. Unlike mice, these $CD27^{+}$ marginal zone B cells are re-circulating, have a diversified repertoire and constitute about 30-40% of the B cells (Dunn-Walters et al., 1995, Tierens et al., 1999, Weill et al., 2009). A murine like- B1 B cell population has not been clearly identified in humans, or in other mammals (Thiriot et al., 2007). Thus, most of our knowledge about B1 B cells is based on studies in mice. It remains to be determined if murine-like Bw B cells are present in humans and other species.

In rabbits and other fellow "GALT" species, there is no well defined classification of mature B cell subsets, due to limitations in the availability of antibody reagents. B cells, for the most part are largely identified by surface IgM or cytoplasmic CD79a that serve as pan B cell markers. Even though a few antibody reagents have been used to delineate B cell subsets, it is not known if these subsets are functionally distinct and parallel the classification scheme used in either mice or humans. Most B cells in rabbits

are $CD5^+$, unlike in mice, where only subsets of B1 B cells express CD5 (Raman and Knight, 1992). However, similar to murine B1 B cells that arise early in ontogeny and express CD43, a subset of IgM^+CD43^+ B cells have been described in the rabbit appendix (Fuschiotti et al., 1997). Interestingly, these B cells are only present during the first few weeks of life, after which they gradually decline with age (Mae Kingzette, personal communication). These cells may likely represent a B1-like lineage that develop in the appendix and migrate to other sites such as the peritoneal cavity.

In sheep, a mAb with unknown specificity, called BAQ44A is expressed at high levels on all B cells in the peripheral tissues. In the ileal PP, only about 5-18% of the B cells bind to BAQ44A and these B cells express high levels of IgM, while the remaining B cells are $IgM^{lo}BAQ44^-$. Consequently, BAQ44A in sheep is considered a marker for mature B cells that emigrate from the primary lymphoid organ, ileal PP (Hein et al., 1989). Gupta et al.(1998) used a different set of markers and described two distinct subsets of B cells. Using antibodies to the complement receptors, CD21 (CR2) and CD11b (CR3), they identified $CD11b^+CD21^-$ and $CD11b^-CD21^+$ populations. The $CD11b^+$ cells were largely non-recirculating, expressed high levels of IgM and localized predominantly in the splenic marginal zone. On the other hand, the $CD11b^-$ B cells were recirculating and expressed low levels of IgM. Although, it is not known how these subsets differ functionally, these findings suggest that murine B1 and B2-like and/or MZ-like lineage cells may exist in sheep as well.

Based on the expression of surface CD2, porcine B cells are classified into two subsets: $CD2^+$ and $CD2^-$ (Sinkora et al., 1998). Analysis of the BM and peripheral blood compartments revealed that CD2 was expressed on B lineage precursors and was either absent or expressed at low levels on recirculating B cells. This pattern of expression is analogous to that of murine HSA and suggests that CD2 in swine distinguishes immature and mature B cells. During fetal and neonatal life, most of the B cells are $CD2^+$ and are gradually replaced with $CD2^{lo/-}$ B cells, consistent with the appearance of mature B cell during ontogeny. Interestingly, these changes in the proportions of $CD2^+$ and $CD2^-$ B cell subsets are dependent on the presence of intestinal microbiota. Germ-free piglets have fewer $CD2^-$ B cells in the periphery when compared to age-matched conventional piglets (Sinkora et al., 1998).

In summary, B cells, when first identified were thought to be a homogenous population of Ig bearing cells (Wilson and Nossal, 1971). Now, based on flow cytometric and functional assays, we have discovered several subsets of B cells. Why do we have so many B cell types? What purpose do they serve in different locations? Our innate immune system act as first line of defense and plays a crucial role in alerting the adaptive immune system to danger. B1 B cells, due to their ability to secrete natural IgM and respond in a T cell independent manner can be considered as part of the innate immune system. Since they are found in tissues other than the peritoneal cavity, they can be considered as a mobile arm of innate immunity. MZ B cells, which share some similarities with B1 B cells, serve as a local defense when pathogens invade the blood

and reach the spleen. Based on these criteria, the conventional B 2 B cells that enter the GCs to form memory B cells can be considered as part of the adaptive immune system. Thus, locations along with distinct competencies of the different B cell subsets serve to constitute an effective humoral immune system.

Concluding remarks

The advent of recombinant DNA technology in the early 1970s has played a crucial role in our understanding of the mechanism(s) by which the primary antibody repertoire is generated. Later, the advent of monoclonal antibodies and technologies to genetically manipulate the mouse, contributed to our understanding of the development and function of B cells. The availability of numerous antibody reagents in mice has helped identify and characterize several phenotypically and functionally distinct B cell subsets. How these distinct B cell populations are generated and maintained in the periphery is now an ongoing area of research. Understanding how the peripheral B cell compartment is maintained may ultimately lead to development of therapeutic strategies in the elderly where B cell immunity is compromised.

The current working model of B cell development in rabbit was derived largely from studies aimed at understanding the mechanism and site(s) for generation of the antibody repertoire. According to the model, B cells develop early in ontogeny and migrate to GALT where microbial antigens stimulate their proliferation and diversification (Knight and Winstead, 1997). While the requirement of commensal bacteria for this early phase of development has been directly demonstrated using a germ-

free appendix model (Rhee et al., 2004), the mechanism(s) that regulate B cell expansion and diversification in GALT, and maintenance of peripheral B cells in adults is not known. For my dissertation, I focused on determining the cellular signals that are required to promote the proliferative expansion of B cells in GALT, and also the role of BAFF during peripheral B cell development.

CHAPTER TWO

MATERIALS AND METHODS

Rabbits

Rabbits were from the colony maintained by K. L. Knight at Loyola University Chicago. Adult rabbits used in this study ranged from 4 months to 2 years of age. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of Loyola University Chicago, Maywood, IL.

Thymectomy and anti-CD4 mAb treatment

For depletion of T cells, newborn rabbits were thymectomized by Dr. P. Sethupathi on day 1 after birth. On days 2 and 5 after birth, the thymectomized rabbits were injected s.c. with anti-CD4 mAb [5 mg/kg body weight (wt)] (clone Ken4) and sacrificed on day 8 after birth. The anti-CD4 mAb was purified by passed Ken4-secreting hybridoma supernatant over a protein G sepharose column.

Cyclosporine A

For depletion of T cells, newborn rabbits were injected (s.c.) daily with cyclosporine A (CsA) (LC Laboratories, Woburn, MA) at the following doses: 0.4mg/kg

body wt on day 1 after birth, followed by 15mg/kg body wt from days 2 to 5. Rabbits were sacrificed on day 6 after birth.

Cobra venom Factor

For depletion of C3 in newborn rabbits, two i.p. injections of Cobra venom factor (CVF) (Calbiochem, San Diego, CA) (0.5mg/kg body wt) were given at 24 and 48 h after birth. Rabbits were sacrificed on day 6 or 7 after birth.

Recombinant adenovirus

Adenoviral (Ad) constructs expressing TACI-Ig (extracellular portion of human TACI fused to human Fcγ) and mouse Fcγ (as control) were kindly provided by Dr. Tong Zhou (University of Alabama at Birmingham, Birmingham, AL) (Liu et al., 2004). Rabbit CTLA4-Ig in pCDNA3 vector was kindly provided by Dr. David Dichek, University of Washington School of Medicine, Seattle, WA. The first two cysteine residues in the rabbit Fcγ portion (CH2 and CH3) of CTLA4-Ig were mutated into serines by the Dichek laboratory. I PCR amplified the Ig portion from this construct using primers: OS-Fcgamma-BAM 5'-atggatccccctcgacatccag-3', OAMycFc-H3-1 5'-tcttctgagatgagttttgttctttacccggaga-3' and OAMyc-Fc-H3-2 5'-ataaagctttcacagatcctcttctgagatgag-3' in order to introduce a myc tag after the Ig . The PCR amplified Ig-myc was then cloned in frame with CTLA4 in pGEM-T vector (Promega, Madison, WI) and then subcloned into CMV-shuttle vector (Qbiogene, Carlsbad, CA) following digestion with XhoI and HindIII restriction enzymes. For constructing CD40-Ig, the extracellular portion of human CD40 was PCR-amplified from

Raji cDNA (using OS HuCD40XhoI 5'-actcgagaccatggttcgtctgcctctgcag-3' and AS HuCD40BamH1 5'-tggatc cccgatcctggggaccacagacaac3') and cloned into CMV-shuttle vector, in frame with rabbit Ig-myc. Similarly, the extracellular portion of rabbit CD21 (CR2) was PCR-amplified from appendix cDNA (using OSrCD21Xho 5'-actcgaggccgccaccatgggcgccgcg-3'ASrCD21Bam 5'- tggatccccccttcattgcaagaaatgtt-3') and cloned into the CMV-shuttle vector in frame with rabbit Fcγ. Following homologous recombination and integration of CTLA4-Ig, CD40-Ig or CD21-Ig into the adenoviral genome, I screened and selected recombinant clones as described by the manufacturer (Qbiogene, Carlsbad, CA). Adenoviral constructs expressing CTLA4-Ig, CD40-Ig, and CD21-Ig (5μg DNA), were transfected into QBI-293A cells (Qbiogene, Carlsbad, CA) using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA). Viral particles were isolated after multiple freeze-thaw cycles and purified using cesium chloride gradients. Virus was titered using the 50% TCID method (Qbiogene, Carlsbad, CA) and stored at -80^{o}C. Recombinant viral particles (10^{10} in 0.3ml PBS) were injected i.p. into rabbit pups within 48 hours of birth and the rabbits were sacrificed 7-10 days later.

Immunization and ELISA

To determine the efficacy of CTLA4-Ig *in vivo*, rabbits neonatally injected with Ad-CTLA4-Ig were re-injected with AdCTLA4-Ig at 2 and 5 weeks of age and 3 days after the 2 week injection, they received 0.5mg Bovine Gamma Globulin (BGG) in Complete Freunds Adjuvant (s.c.). After the 5 week injection of AdCTLA4-Ig, they received a secondary immunization of 0.5mg BGG in Incomplete Freunds Adjuvant.

Serum was harvested on day 7 after the primary and day 10 after the secondary immunization and anti-BGG IgM and IgG levels were determined by ELISA using anti-rabbit IgM (clone 367) or anti-rabbit IgG (clone 359; BD Biosciences, San Jose, CA) coated microtiter plates. The ELISA was developed with goat anti-rabbit H&L chain-HRP (Jackson ImmunoResearch, Westgrove, PA) plus ABTS (Sigma Aldrich, St. Louis, MO) as substrate. The relative levels of serum IgM and IgG in AdCTLA4-Ig-treated and control PBS-treated rabbits were determined from a linear portion of the dilution curves.

Flow cytometry and immunohistochemistry

Cross-reactive and rabbit-specific antibodies used are shown in tables 1 and 2, respectively. Indirect reagents that were used are as follows: Dylight 649, 488, or FITC conjugated goat Fab anti-mouse IgG, and streptavidin FITC, DTAF, PE or APC (Jackson ImmunoResearch, Westgrove, PA). Rabbit rBAFF, human TACI-Ig, CD40-Ig, and CTLA4-Ig were biotinylated (1mg) using NHS-LC biotin (Pierce Biochemicals, Rockford, IL). Biotinylated CTLA4-Ig was used as a reagent to detect surface CD80 and CD86. Biotinylated PNA was purchased from Sigma, St. Louis, MO. For analysis of transitional B cells, multicolor flow cytometry (3, 4 or 5 color) was performed by gating on $CD24^{hi}$ cells that were either $CD21^{lo}/CD21^{+}$ or IgM^{lo}/IgM^{+}. As a negative control, cells were stained with isotype control antibodies. All flow cytometry data were acquired with FACSCanto or FACSCantoII or FACSAria (BD Biosciences, San Jose, CA), gated on live lymphocyte-sized cells on the basis of forward and side scatter, and analyzed

using FlowJo software (Tree star, Ashland, OR). All FACs plots using fluorescent reagents are depicted on a logarithmic scale except where indicated.

For flow cytometric analysis of commensal bacteria, luminal contents were flushed from rabbit appendix with 1XPBS, 5% fetal calf serum (FACs buffer), and debris was removed by centrifugation (300 X g) for 1 min. Bacteria were pelleted by centrifugation (2800 X g) for 15 min and resuspended in buffer. 25-50µl bacteria (representing a pellet of ~1-2 $mm^{3)}$ were stained in Eppendorf tubes with the appropriate Abs and analyzed as described above.

For immunohistochemistry, acetone-fixed cryosections (7-8µm) were blocked with goat serum (5-10%) and then stained with primary Abs (Tables 1&2) and indirect reagents: Cy2- or Cy3-conjugated streptavidin and Cy2- or Dylight 549-conjugated goat (Fab) anti-mouse IgG (Jackson ImmunoResearch,Westgrove, PA). Slides were viewed under a Leica DM IRB microscope (Leica Microsystems, Brannockburn, IL) and images captured using the MagnaFire 2.1C digital camera system (Optronics, Goleta, CA). The frozen germ-free (GF) appendix tissues and GALTless spleen tissues used for immunohistochemistry were obtained from rabbits previously described (Vajdy et al., 1998, Rhee et al., 2004, Severson et al., 2010).

Table 1: Cross-reactive antibodies

Antibody	Specificity	Clone	Vendor
CD10	Human	CB-CALLA	eBiosciences, Inc., San Diego, CA
CD20[a]	Human	B9E9	Santa Cruz Biotechnology, Inc., Santa Cruz, CA Immunotech, Marseille Cedax, France
CD21	Human	BL13	Immunotech, Marseille Cedax, France
CD23 [a]	Human	9P25	Immunotech, Marseille Cedax, France
CD24	Mouse	M1/169	eBiosciences, Inc., San Diego, CA BD Biosciences, San Jose, CA
CD27 [a]	Human	LT27	AbD Serotec, Oxford, UK
CD38	Human	IB6	Kindly provided by Dr. Malavasi, University of Turin, Italy
CD62L	Human	LAM-1	Kindly provided by Dr. Tedder, Duke University, Durham, NC
CD79a [a]	Human	HM47	BD Biosciences, San Jose, CA
CD90	Human	5E10	BD Biosciences, San Jose, CA
Ki-67 [a]	Human	B56	BD Biosciences, San Jose, CA
BCL6 [a]	Human	BL6.02 (PG-B6p)	Thermo Fisher Scientific, Fremont, CA

Antibody	Specificity	Clone	Vendor
Caspase3	Human/Mouse	C92-605	BD Biosciences, San Jose, CA
BR3	Human	pAb [b]	R&D Systems, Inc., Minneapolis, MN
BAFF [a]	Human	pAb [b]	R&D Systems, Inc., Minneapolis, MN

[a]Also used for immunohistology

[b]Polyclonal antibody raised in Goat

Table 2: Rabbit specific antibodies

Antibody	Clone	Vendor
IgM [a]	367	BD Biosciences, San Jose, CA
IgA	102	BD Biosciences, San Jose, CA
IgG	359	BD Biosciences, San Jose, CA
L Chain [a]	pAb [b]	KLK Stock
CD1b [a]	LAT-3	Kindly provided by Dr. Steward Sell, Albany Medical College, Albany, NY
CD3 [a]	PC3/188A	Spring Valley Laboratories Inc., Woodline, MD
CD4 [a]	Ken4	BD Biosciences, San Jose, CA
CD9 [a]	MM2	Antigenix America, Huntington Station, NY
CD11b	198	Antigenix America, Huntington Station, NY
CD11c [a]	3/22	R&D Systems, Inc., Minneapolis, MN
CD14	K4	Antigenix America, Huntington Station, NY
CD25	Kei-α1	BD Biosciences, San Jose, CA
CD43	L11/43	Antigenix America, Huntington Station, NY
CD44	W4/86	Chemicon International, Temecula, CA
MHC II	2C4	BD Biosciences, San Jose, CA
C3 [a]	pAb [b]	SouthernBiotech, Birmingham, AL
MΦ [a]	RAM11	Dako, Carpinteria, CA

[a]Also used for immunohistology

[b]Polyclonal antibody raised in Goat

In situ hybridization

Paraformaldehyde (4%) fixed tissue sections (10-12µm) were hybridized with 10-30ng/ml digoxigenin-labeled riboprobes, 70^{o}C, 20 hr, in a humidified chamber as described by Hanson and Lanning (2008). Hybridized sections were stringently washed and incubated with HRP-F(ab')-anti-digoxigenin followed by biotinyl tyramide (DAKO), and then AP-F(ab')-anti-biotin (DAKO, Carpinteria, CA). Sections were developed with BCIP/NBT (Vector Labs, Burlingame, CA) and examined with the Leica DM IRB microscope as described above. ***Riboprobe preparation:*** Approximately 500-800 bp PCR product from each gene of interest (BAFF, APRIL, AID, and CD40L) was cloned into the pGEM-T vector (Promega, Madison, WI). Sense and anti-sense *in vitro* transcription templates were generated by PCR amplification from each plasmid, using the appropriate gene-specific forward or reverse primer, as described below, with a plasmid-specific primer containing either the T7 or SP6 RNA polymerase promoter (T7 Univ: 5'-agtgaattgtaatacgactcactataggg-3' and SP6 Univ: 5'-cgccaagctatttaggtgacactatagaatac-3'). 200ng of purified PCR product was used as a template in *in vitro* transcription reactions containing RNA labeling mix with digoxigenin-UTP (Roche, Mannheim, Germany) and T7 or SP6 RNA polymerase (Fisher Scientific, Pittsburg, PA). Plasmid template was digested with RNase-free DNase I (Fermentas Inc., Hanover, MD) and the reactions were stopped by addition of 0.2M EDTA. RNA transcript integrity and concentration were assessed by electrophoresis on denaturing polyacrylamide gels (8M Urea and 5% acrylamide) run in TBE buffer. GenBank accession numbers for nucleotide sequences

used to prepare riboprobes and primers used are as follows: BAFF (ABP01347; nucleotides 417-873) (OSRbBAFF 5'-cttaaggcaactcccacaccttctct-3'and OAaHuBAFF 5'-cctaggagtgtcgtcaaagttacgtg-3'), APRIL (EF494239; nucleotides 1-753) (RbAPRIL KpnI F 5'- ggtacccgggctcatgcca-3' and RbAPRIL BamHI R 5'- tccaggcagtttcacaaaccccaggaag-3'), AID (AY928183; nucleotides 1-577) (RbAID F 5'-atggacagcctcttgatgaa-3' and RbAID R 5'- tcaaartcccaaagttacgaaatgc-3') and CD40L (nucleotides 1-785) (OSrbCD40L 5'-atgatcgaaacgtacagccaacct-3' and ASrbCD40L 5'-tcagagtttgagtaagccaaatga-3').

Quantitative real-time PCR (qPCR)

Total RNA was isolated from 55D1 B cells, FAC-Sorted IgM^+ B cells, BM $CD11b^+$ cells (gated on side scatter high cells), and peripheral blood (PB) $CD14^+$ monocytes using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized (starting with100ng RNA for each sample) using the Superscript III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). BAFF and β-actin (endogenous control) was PCR-amplified with primers: rb BAFF Forward 5'-tgattgcag acagtgacacaccga-3', rb BAFF Reverse 5'-aggtacccggtttctttgaccact-3' and rb β-actin Forward 5'-agatgtggatcagcaagcaggagt-3', rb β-actin Reverse 5'-agccatgccaatctcgtctcgttt-3', respectively on an Applied Biosystems 7300 Real time PCR System (Applied Biosystems, Foster City, CA). The relative level of BAFF mRNA normalized against β-actin was analyzed using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001).

Cloning and expression of BAFF and APRIL

Soluble BAFF (sBAFF) (aa134-284) was PCR-amplified from rabbit appendix cDNA (OS RbsBAFF 5'-aagcttgttgagggtgtggaagag-3' and AS RbsBAFF 5'-ctcgagcaacaacttcagtgcacc-3') and cloned into pET-24b vector (Novagen, Madison, WI). Recombinant sBAFF was expressed in *Escherichia coli* BL21λ DE3 cells (Invitrogen, Carlsband, CA) and purified from the insoluble fraction using a Ni^{2+}-NTA agarose column (Qiagen, Valencia, CA). sBAFF was dialyzed against refolding buffer (0.05M glycine,0.03M NaOH, 0.4M L-arginine, 1mM dithiothreitol, pH 10.0) for 24 hrs at 4^{o}C, followed by dialysis in PBS at 4^{o}C (all chemicals purchased from Sigma, St Louis, MO). Full length BAFF (fl BAFF) was PCR-amplified from appendix cDNA (OS FLRbBAFF 5'-ctcgaggccgccaccatggatgactccacg-3' and AS FLRbBAFF 5'-aagcttcaacaacttcagtgcaccgaagaa-3') and cloned into pEGFP-N1 vector (Clonetech Laboratories, Palo Alto, CA), such that BAFF was in-frame with EGFP. Chinese hamster ovary (CHO) cells were transfected with BAFF-GFP fusion construct and cultured in the presence of 50μM ChloroMethyl Ketone (CMK) (Calbiochem, San Diego, CA) for 3 days to minimize shedding of BAFF. Only GFP positive cells were analyzed by flow cytometry for binding to biotinylated TACI-Ig. Soluble APRIL (sAPRIL) (aa105-250) was PCR-amplified from appendix cDNA using primers: OS RbsAPRIL 5'-agatctgcactgcccacccagaaa-3' and ASRbsAPRIL 5'-ctgcagtcacagtttcacaaaccc -3'. The underlined nucleotides in the reverse primer indicate a stop codon. The sAPRIL PCR product was cloned into pDISPLAY vector (Invitrogen, Carlsbad, CA) and transfected

into 293T cells. Transfected cells were cultured in serum-free media and the supernatant containing sAPRIL was harvested after 4-5 days and used in B cell stimulation assays. The nucleotide sequence of all constructs was confirmed by nucleotide sequence analysis.

Western blot

55D1 B cells, FAC-sorted IgM^+ B cells, and BM $CD11b^+$ cells (10^6) were lysed in buffer containing 4% SDS and 3% DTT and electrophoresed on 12% SDS-PAGE gel. Proteins were transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MD) and probed with a cross-reactive goat anti-human BAFF antibody (ref) (Antigenix America, Huntington Station, NY), followed by biotinylated rabbit Fab anti-goat IgG (Rockland, Gilbertsville, PA), streptavidin-HRP (Jackson ImmunoResearch, Westgrove, PA) and SuperSignal® substrate (Pierce, Rockford, IL). To demonstrate equal loading of lysates, blots were stipped and re-probed with anti β-actin Ab (clone AC-15; Sigma, St. Louis, MO).

Proliferation and survival assays

For BAFF and APRIL co-stimulatory experiments, 10^5 splenocytes and appendix cells (in 100µl) were cultured with anti-Ig (10µg/ml) [goat (F(ab') anti-rabbit IgG (H+L); Jackson ImmunoResearch Laboratories] and varying concentrations of soluble BAFF or supernatant containing soluble APRIL. Proliferation was assessed by ^{3}[H]-thymidine (1µCi/well) (Amersham Biosciences) incorporation using a liquid scintillation counter (PerkinElmer, Inc). As a positive control for proliferation, splenocytes and appendix cells

were cultured with irradiated murine CD40L-transfected CHO cells in a 100:1 ratio, respectively and pulsed with ^{3}[H]-thymidine.

For survival assays, appendix B cells were depleted of $CD4^{+}$T cells using anti-CD4 and anti-mouse Ig coated magnetic beads (MACS; Miltenyi Biotech). Enriched B cells (10^{6}/ml) were cultured in either media alone or BAFF (3µg/ml) or heat inactivated BAFF (95^{o}C for 30 minutes). Live cells in culture were enumerated after 48 and 96 hrs.

To assess BAFF-binding to *in vitro* activated B cells, mononuclear cells from peripheral blood, spleen and appendix were cultured in the presence of anti-Ig (10µg/ml) and CD40L-expressing CHO cells as described above. After 3 days in culture, live B cells were analyzed by flow cytometry for BAFF-binding.

Apoptosis and cell cycle analysis

FAC-sorted splenic T1 and mature B cells were cultured with anti-Ig (10µg/ml) [goat (F(ab') anti-rabbit IgG (H+L); Jackson ImmunoResearch, Westgrove, PA] for 12-15 hours, followed by staining with propidium iodide (PI) and FITC-annexin V (BD Biosciences, San Jose, CA) and analyzed by flow cytometry as described above. Similarly, FAC-sorted appendix $CD23^{+}$ and $CD23^{-}$ B cells were cultured with anti-Ig (10µg/ml) for 6, 15 and 25 hours, followed by staining with PI and annexin V at each time point to enumerate the frequency of apoptotic cells.

For cell cycle analysis, FAC-sorted splenic T1 and mature B cells (and $CD27^{+}$ and $CD27^{-}$ B cells) were cultured with anti-Ig (10µg/ml) or with irradiated murine

CD40L-transfected CHO cells in a 100:1 ratio, respectively. After 24 h, cells were fixed with cold 70% ethanol, treated with RNase (50µg/ml), stained with PI (50µg/ml) and analyzed by flow cytometry (FlowJo software).

Ig secretion assay

FAC-sorted $CD27^+$ and $CD27^-$ B cells (10^4) were cultured (in 200µl) with irradiated murine CD40L-transfected CHO cells in a 100:1 ratio, respectively, and human IL4 (100ng/ml) (R&D Systems Inc., Minneapolis, MN). The culture supernatants were harvested after 7-8 days and the total amount of Ig secreted was measured by ELISA using anti-L Chain antibody.

CHAPTER THREE

RESULTS

CO-STIMULATORY MOLECULES REQUIRED FOR PROLIFERATIVE EXPANSION OF B CELLS IN GALT

In mammals that use GALT to generate the primary antibody repertoire, B cells that seed GALT, expand in number, form organized follicles, and somatically diversify the Ig genes. In rabbits, using a germ-free appendix model, Rhee et al. (2004) demonstrated that commensal bacteria are required for the proliferative expansion of B cells and somatic diversification of Ig genes. The mechanism(s) by which commensal bacteria regulate these processes in GALT is not known. Little is known about the cellular interactions, cytokines and other factors that are required to sustain B cell proliferation and survival in GALT.

To understand the early stages of B cell development in rabbit GALT, I investigated some of the mechanism(s) by which B cells may be induced to proliferate. B cells can be stimulated both in a T cell dependent and T cell independent manner, depending on the nature of the antigen. In T cell dependent stimulation, signal 1 is delivered to B cells when BCR is cross-linked after binding antigen and signal 2 is delivered following a direct contact with T cells via the CD40/CD40L interactions and

other T cell derived cytokines. In contrast, T cell independent stimulation of B cells can occur through the cross-linking of BCR by highly repetitious molecules such as bacterial cell wall polysaccharides, or by bacterial superantigens that bind the framework region of the BCR. In addition, B cells can also be stimulated through other receptors like the toll receptors, mitogen receptors, complement and BAFF receptors in a T cell independent manner.

I proposed that B cells in GALT are stimulated by bacteria or bacterial products in a T cell independent manner. Bacteria may regulate the expression of co-stimulatory molecules, cytokines, and other factors that are required for B cell proliferation and somatic diversification of Ig genes. I do not think that B cell development in GALT is initiated by an antigen-specific (immune) response to bacterial antigens. Rather, I hypothesized that B cells are stimulated in a polyclonal manner. Thus, if B cell stimulation is not antigen-driven, then one would predict that T cell help would not be required during the early phase of B cell development in GALT. This is the case in sheep. Reynaud et al. (1995) thymectomized a lamb at 70 days gestation and achieved a peripheral T cell depletion up to 90% and found normal levels of somatic diversification of Ig genes in this animal. Thus, it appears that T cells are not required for the development of primary antibody repertoire in sheep. Similarly, I hypothesize that T cells are not required for B cell development in the rabbit GALT.

Thymectomy and anti-CD4 mAb treatment to deplete T cells

One approach to directly test if T cells are required for B cell development in GALT is to develop T cell deficient rabbits and determine if B cells proliferate and somatically diversify the Ig genes. Because thymectomy combined with high dose anti-CD4 mAb treatment in both mice and cattle proved useful in depletion of $CD4^+$ T cells from both circulation and secondary lymphoid organs (Cobbold et al., 1984, Valdez et al., 2001), I attempted to deplete T cells in neonatal rabbits using a similar strategy. To evaluate the effect of anti-CD4 mAb treatment from thymectomy alone, I injected anti-CD4 mAb into one of the two rabbits that were thymectomized at birth, and sacrificed them along with a litter-mate control on day 9 after birth. To determine if T cells were depleted by this treatment, I analyzed the frequency of $CD4^+$ T cells and IgM^+ B cells in the spleen and appendix and found a similar frequency of T cells in the spleen of control and thymectomized rabbit, and only a slight reduction in the frequency of T cells in the thymectomy + anti-CD4 mAb treated rabbit (Fig 1A). To functionally evaluate the effect of thymectomy + anti-CD4 mAb treatment on the residual T cells, I stimulated splenocytes from control and treated rabbits with a T cell mitogen, ConA, and measured proliferation by [^{3}H]-thymidine incorporation. I found approximately a 2-fold decrease in thymidine incorporation in splenocytes from the treated rabbit when compared to splenocytes from the control rabbit (Fig 1B). This decrease in [^{3}H]-thymidine incorporation likely reflects the nearly 2 fold reduction in the number of T cells in the spleen of the thymectomy + anti-CD4 mAb treated rabbit. I conclude that neither

thymectomy nor thymectomy in combination with anti-CD4 treatment was effective in eliminating peripheral T cells.

Similar to the spleen, in the appendix, I found a similar frequency of $CD4^+$ T cells in the control and thymectomized rabbit (data not shown). However, unlike spleen, I found no decrease in the frequency of $CD4^+$ T cells in the appendix of thymectomized + anti-CD4 mAb treated rabbit (Fig 1C). To further confirm this observation, I analyzed appendix sections by immunohistochemistry, and was surprised to find a lack of well-defined T cell areas (TCAs) in the appendix of thymectomized + anti-CD4 mAb treated rabbit (Fig 1D, *upper panel*). Upon closer examination (at a higher magnification), I found $CD3^+$ T cells scattered throughout the appendix, and found some cells near the follicle associated epithelium (FAE) (Fig 1D, *lower panel*). Because CD4 on T cells also serves as an adhesion molecule (Killeen et al., 1993), I think administration of anti-CD4 likely interfered with the homing and/or retention of T cells in the TCAs of the appendix. To determine if this altered distribution of T cells in the appendix has any effect on B cell proliferation in GALT, I stained the appendix for Ki-67, a proliferation antigen, and found normal expression of Ki-67 in the basolateral region of B cell follicles (Fig 1E). Taken together, although thymectomy + anti-CD4mAb treatment altered homing/localization of appendix T cells in this experiment, it did not prove effective in depleting T cells. Further, thymectomy alone was also not effective in depleting T cells. Therefore, I looked for alternate strategies to address if T cells are required for B cell development in GALT.

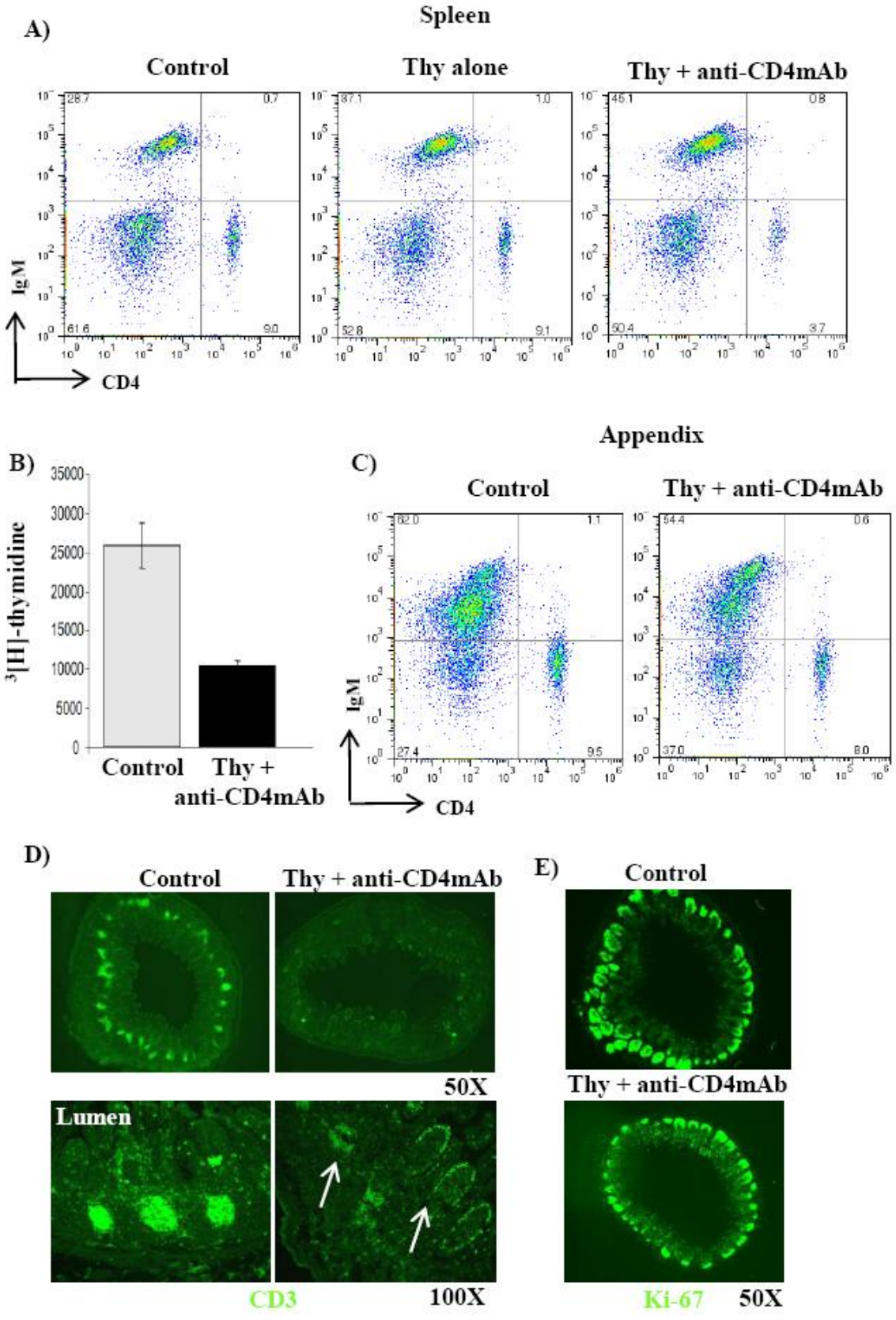

A)
Spleen
Control
Thy alone
Thy + anti-CD4mAb
IgM
CD4
B)
[3H]-thymidine
Control
Thy + anti-CD4mAb
C)
Appendix
Control
Thy + anti-CD4mAb
IgM
CD4
D)
Control
Thy + anti-CD4mAb
50X
Lumen
CD3
100X
E)
Control
Thy + anti-CD4mAb
Ki-67
50X

Figure 1: Analysis of the T cell compartment in thymectomized and thymectomized + anti-CD4 mAb treated rabbits. Flow cytometric staining for IgM and CD4 in the spleen (A) and appendix (C). B) Splenocytes (10^5) from the control and treated rabbits were stimulated with ConA (3µg/ml) for 3 days and proliferation was assessed by [^{3}H]-thymidine incorporation. D) and E) immunofluorescent staining of the appendix for CD3 and Ki-67, respectively. Arrows indicate T cells lining near the FAE and in small clusters. Magnification = 100X or 50X.

Administration of CsA to deplete T cells and/or inhibit their activity

Cyclosporine (CsA) is a widely used immunosuppressive agent. Investigations into its mechanism(s) of action revealed that it inhibits development of T cells in the thymus and the function of T cells in the periphery. Jenkins et al. (1988) demonstrated that administration of CsA in mice inhibited the development of mature single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) TCR- $\alpha\beta^+$ thymocytes. While investigating the biology of Cottontail rabbit papillomavirus, Hu et al. (2005) administered CsA into adult rabbits and found a significant reduction in the percentage of $CD4^+$ and $CD8^+$ T cells in the peripheral blood, presumably due a block in T cell development in the thymus. Lastly, while investigating the mechanisms regulating human CD40L gene expression, Fuleihan et al. (1994), found that CD40L expression was inhibited by pretreatment of T cells with CsA. Consequently, they suggested that patients receiving CsA may be deficient in CD40L-dependent T cell help (Fuleihan et al., 1994).

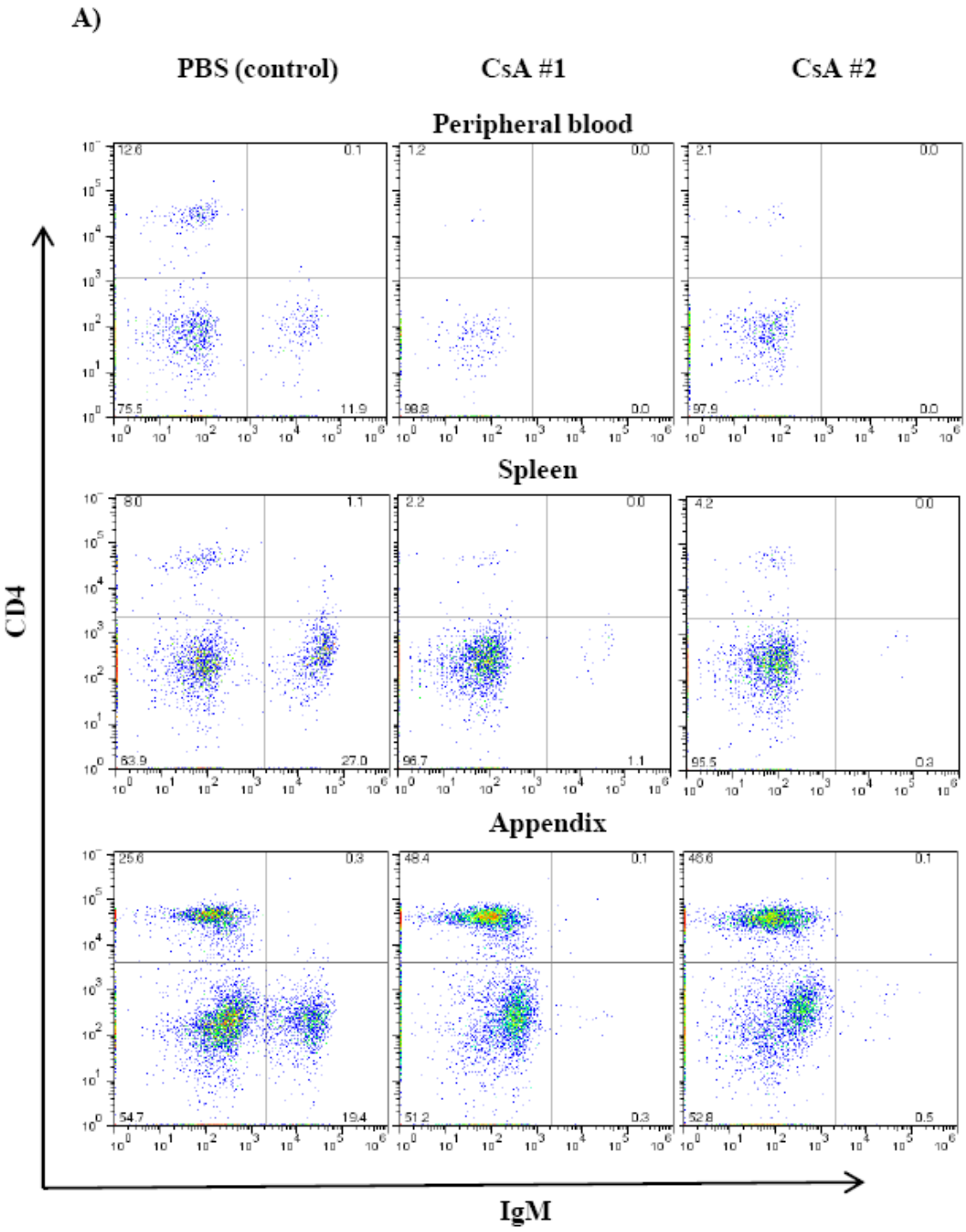
Based on these reports, I reasoned that administering CsA into newborn rabbits may serve as an alternate strategy to deplete T cells and/or inhibit T-B cell interaction. Therefore, I administered CsA into two newborn rabbits on day 1after birth and continued injecting them every day until day 5, and sacrificed the pups along with a litter-mate control on day 6 after birth. By flow cytometry, I found a significant reduction in the frequency of $CD4^+$ T cells in the peripheral blood (PB) and spleen of CsA treated rabbits. Surprisingly, I also did not find any IgM^+ B cells in these animals (Fig 2A). Further, unlike spleen and PB, $CD4^+$ cells were present in the appendix of CsA treated

rabbits (Fig 2A, *lower*). While I expected to find a decrease in the number of T cells upon CsA treatment, I did not expect to find an almost complete absence of the B cell compartment. To confirm the results of the flow cytometry experiment, I analyzed the spleen and appendix tissues from CsA treated rabbits by immunohistochemistry. Consistent with the flow cytometric analysis, I found no B cell follicles either in the spleen or appendix of CsA treated rabbits, but I did find $CD4^+$ TCAs in the appendix (Fig 2B).

In conclusion, while attempting to deplete T cells using CsA, I serendipitously discovered that daily administration of CsA in neonates also causes depletion of B cells. Therefore, I sought an alternate strategy to test whether T cell help is required for B cell development in GALT.

Inhibition of co-stimulatory molecules involved in T-B cell interactions

One approach to test if T cells are required for B cell development in GALT is to inhibit T-B cell interactions *in vivo*, by interfering with essential co-stimulatory signals. As described previously, during a cognate T-B cell interaction, activated T cells provide the critical CD40L signal required for B cell activation. Thus, if T cell activation is inhibited, then CD40L will not be upregulated on T cells, resulting in a block in T-dependent B cell activation. T cell activation can be inhibited through the use of a soluble CTLA4 receptor, since CTLA4 can effectively block CD28 mediated costimulation of T cells by a competitive inhibition at the B7 binding site (Linsley et al., 1992).


A)
PBS (control)
CsA #1
CsA #2
Peripheral blood
Spleen
Appendix
CD4
IgM
12.6
0.1
75.5
11.9
1.2
0.0
98.8
0.0
2.1
0.0
97.9
0.0
8.0
1.1
63.9
27.0
2.2
0.0
96.7
1.1
4.2
0.0
95.5
0.3
25.6
0.3
54.7
19.4
48.4
0.1
51.2
0.3
48.6
0.1
52.8
0.5

B)

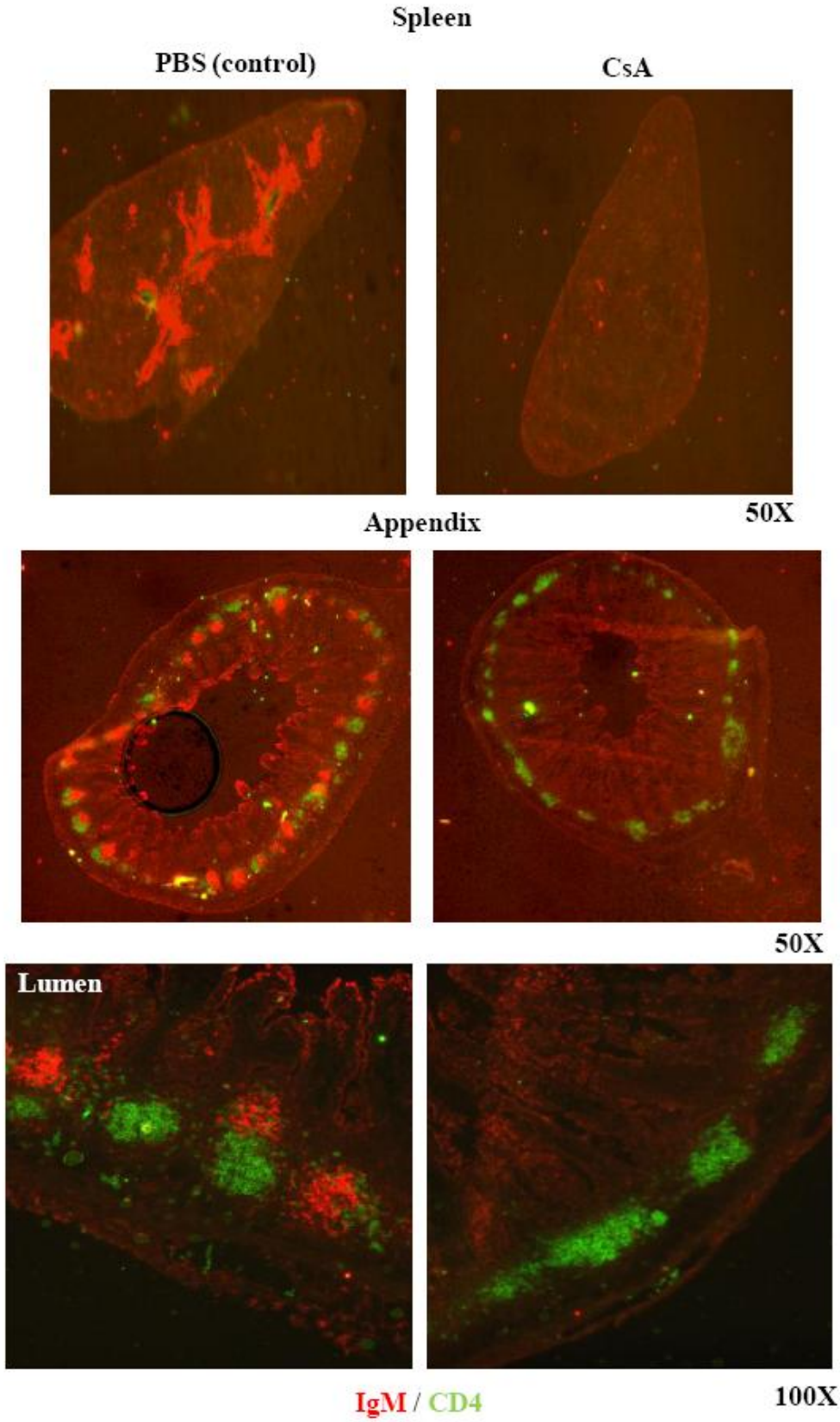


Figure 2: Analysis of the T and B cell compartments in CsA-treated and control (PBS) rabbits. A) Flow cytometry of IgM and CD4 stained cells in the peripheral blood, spleen, and appendix. B) Immunofluorescent staining for IgM and CD4 in the spleen (magnification=50X) and appendix (magnification = 50X and 100X). Data in B) are representative of staining obtained from one of the two CsA-treated rabbits.

To inhibit T cell activation and thereby prevent T cell help, I injected newborn rabbits with a recombinant adenovirus (rAd) expressing soluble CTLA4 (CTLA4-Ig). After 7-10 days, I analyzed the appendix by immunohistochemistry for the presence of follicles with proliferating (Ki-67^{+}) B cells and found that CTLA4-Ig did not inhibit B cell proliferation (Fig 3A). To confirm that the absence of a phenotype in the CTLA4-Ig injected rabbits was not due to insufficient or non-functional CTLA4-Ig, I immunized two rabbits with a T cell dependent antigen (BGG) and found, as expected, a dramatic reduction in both primary IgM and secondary IgG (anti-BGG) Ab titers (Fig 3B). Further, unlike in appendix, CTLA4-Ig inhibited B cell proliferation in the spleen, indicating that CTLA4-Ig is functional *in vivo* and inhibits an immune response to a T cell dependent antigen (Fig 3C).

To determine if the Ig genes in peripheral B cells are somatically diversified in CTLA4-Ig treated rabbits, ideally, I would clone and sequence Ig genes from B cells of 6-8 week-old rabbits, a time period when B cells with a diversified repertoire begin to appear (Crane et al., 1996). However, because the titer of CTLA4-Ig was highest during the first 1 week after injection of rAd, and rapidly dropped thereafter, the presence of somatic diversification at 6 – 8 weeks of age would not help me determine if CTLA4-Ig had inhibited somatic diversification. Consequently, I looked for the presence of Activation-induced Deaminase (AID), an enzyme required for somatic hypermutation, as an indirect measure to assess if B cells will undergo somatic diversification. If AID is expressed in the follicles at 1 week of age, then it is reasonable to assume that those B

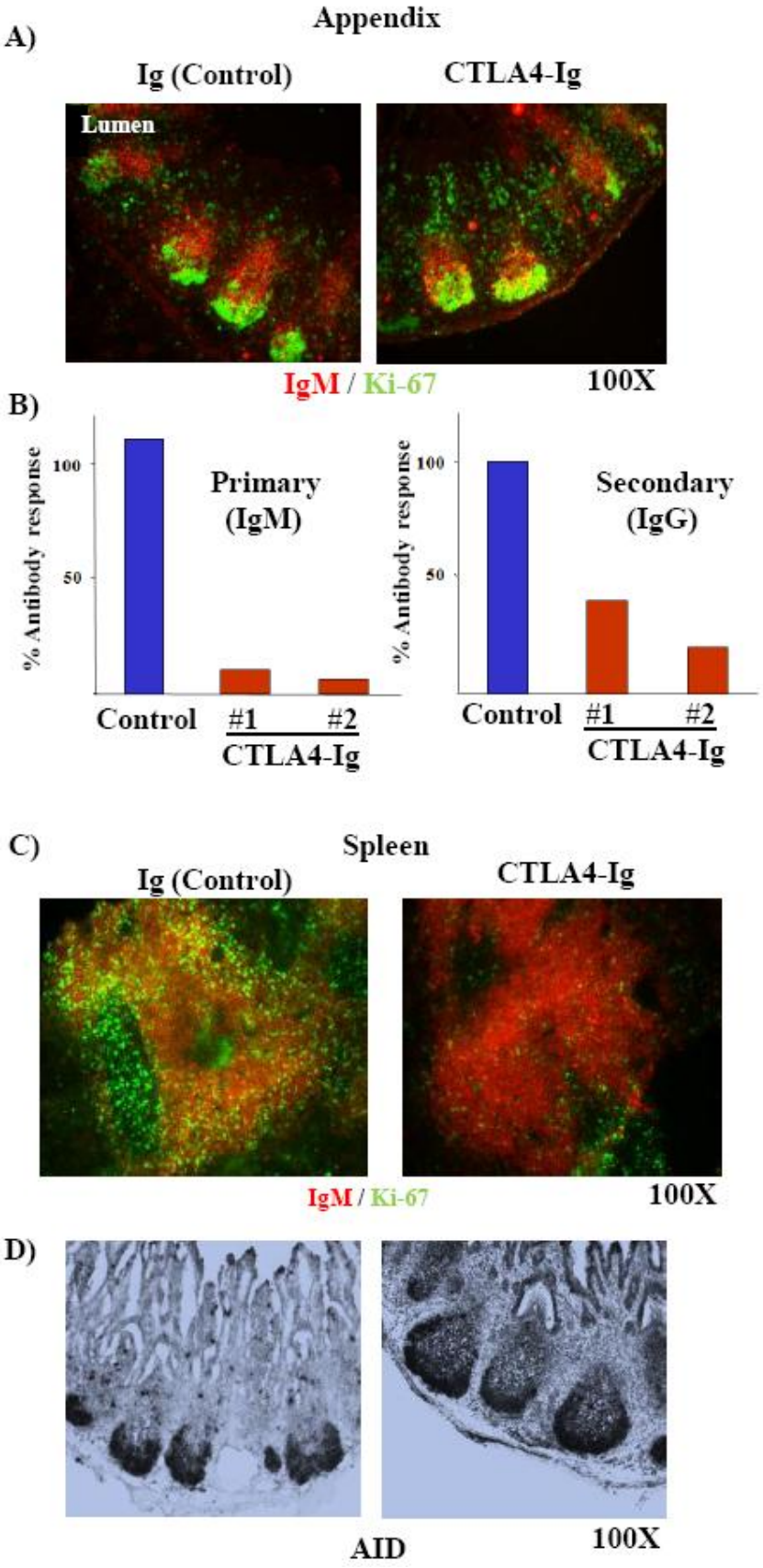

A)
Appendix
Ig (Control)
CTLA4-Ig
Lumen
IgM / Ki-67
100X
B)
% Antibody response
100
50
Primary
(IgM)
Control
#1
#2
CTLA4-Ig
% Antibody response
100
50
Secondary
(IgG)
Control
#1
#2
CTLA4-Ig
C)
Spleen
Ig (Control)
CTLA4-Ig
IgM / Ki-67
100X
D)
AID
100X

Figure 3: Analysis of the B cell compartment in CTLA4-Ig-treated and control (Ig) rabbits. A) Immunohistochemical staining of the appendix with anti-IgM and anti-Ki-67 mAbs. B) Bar graph showing the primary anti-BGG (IgM) (*left*) and secondary anti-BGG (IgG) (*right*) response, compared to the anti-BGG response from an age-matched littermate controls (=100%) as determined by ELISA. #1 and #2 represent data from two AdCTLA4-Ig treated rabbits. C) Immunofluorescent staining of the spleen from one of the immunized rabbits in b) for IgM and Ki-67. D) *In situ* hybridization to detect AID transcripts in the appendix from control and CTLA4-Ig-treated rabbits. Data in A) are representative of three Ig (control) and CD40-Ig treated rabbits. Magnification = 100X.

cells are undergoing diversification. I tested for AID expression in 1 week-old CTLA4-Ig injected rabbits and found AID transcripts in the basolateral region of B cell follicles (Fig 3D), suggesting that somatic diversification of Ig genes can occur in the absence of activated T cells.

To determine if CD40-CD40L interaction is required for the proliferative expansion and somatic diversification of B cells in the appendix, I injected newborn rabbits with rAd expressing soluble CD40 (CD40-Ig) and found that it inhibited B cell proliferation in the appendix (Fig 4A). Compared to control rabbits, I found reduced levels of AID in the basolateral regions (indicated by arrow) of the B cell follicles (Fig 4D). The reduced expression of AID in CD40-Ig-treated rabbits could be due to lack of proliferating B cells in the basolateral region of the follicles. These data indicate that CD40-CD40L interaction is required for B cell development in GALT. Because data from CTLA4-Ig injected animals suggested to me that CD28 mediated activation of T cells is not required for GALT development, I asked what could be the cellular source of CD40L. To address this question, I analyzed young rabbit appendix tissue sections (1 week of age) by immunohistochemistry using purified CD40-Ig as a reagent, to detect CD40L expression pattern. If T cells express CD40L, and are the primary source of CD40L in the appendix, then I expected to find CD40-Ig staining the TCAs. Instead, I found that CD40-Ig stained, albeit weakly, the entire appendix tissue, and not only the TCAs (Fig 4C). Consistent with CD40Ig staining, I noticed a similar localization of CD40L transcripts by *in situ* hybridization (Fig 4D). Although originally described on

activated T cells, CD40L is also expressed on non-lymphoid cells such as epithelial cells, monocytes, dendritic cells, fibroblasts, smooth muscle cells and endothelial cells (Schonbeck and Libby, 2001). Therefore, I think the staining pattern obtained with CD40Ig and CD40L riboprobe could be due to a low level expression of CD40L by a variety of cell types in the appendix.

From these studies, I conclude that activation of T cells via the B7-CD28 pathway is not required for the proliferative expansion and somatic diversification of B cells in GALT, whereas CD40-CD40L interaction is required. Further, based on the distribution of CD40L in the appendix, I suggest that non-T cells may be a cellular source of CD40L.

Role of complement during B cell development in GALT

The importance of the complement system in inflammation is well appreciated; however, increasing evidence supports its important role also in regulation of B lymphocytes (Carroll, 2004, Tew et al., 2001, Rossbacher et al., 2006). The identification and characterization of complement receptors CD21 (CR2) on B cells and CD35 (CR1) on follicular dendritic cells (FDCs) greatly expanded our understanding of how complement acted on B cells (Tew et al., 2001, Fearon and Wong, 1983, Fischer et al., 1998)

accessory cells in the B cell follicles, I reasoned that FDCs may capture and present bacterial antigens to follicular B cells in the form of immune complexes. These interactions may in turn promote B cell proliferation and expansion in GALT. To address whether B-FDC cell interactions via surface CD21 and its ligands are required for GALT development, I injected newborn rabbits with a rAd expressing soluble CD21 (CD21-Ig) and analyzed the appendix by immunohistochemistry after 7-10 days. Upon CD21-Ig treatment, I found little to no Ki-67 expression in the appendix (Fig 5A), indicating that signaling through CD21 is required for B cell development in GALT. As an alternate approach, I administered cobra venom factor (CVF) into newborn rabbits and depleted C3 *in vivo* (Fig 5B). Using a polyclonal anti-C3 antibody in western blot analysis, I found no C3 and C3d proteins in the sera of CVF-treated rabbits, while I readily detected these proteins in control rabbits (Fig 5B). Similar to CD21-Ig treatment, I found that B cell proliferation in the appendix was inhibited following CVF treatment (Fig 5A, *right*). From these experiments, I conclude that complement is required for B cell proliferation in GALT.

How is complement activated in GALT? Do commensal bacteria play a role in regulating complement expression and localization in GALT? To address this question, I examined germ-free (GF) appendix and found no C3 deposition, whereas in conventional appendices, I found C3 readily localized in the B cell follicles (Fig 6A). To determine if colonization of the intestine by commensal bacteria induced C3 expression in the follicles, I examined GF appendices into which select bacteria were introduced (Fig 6B). The Knight lab previously demonstrated that GALT development was induced in GF

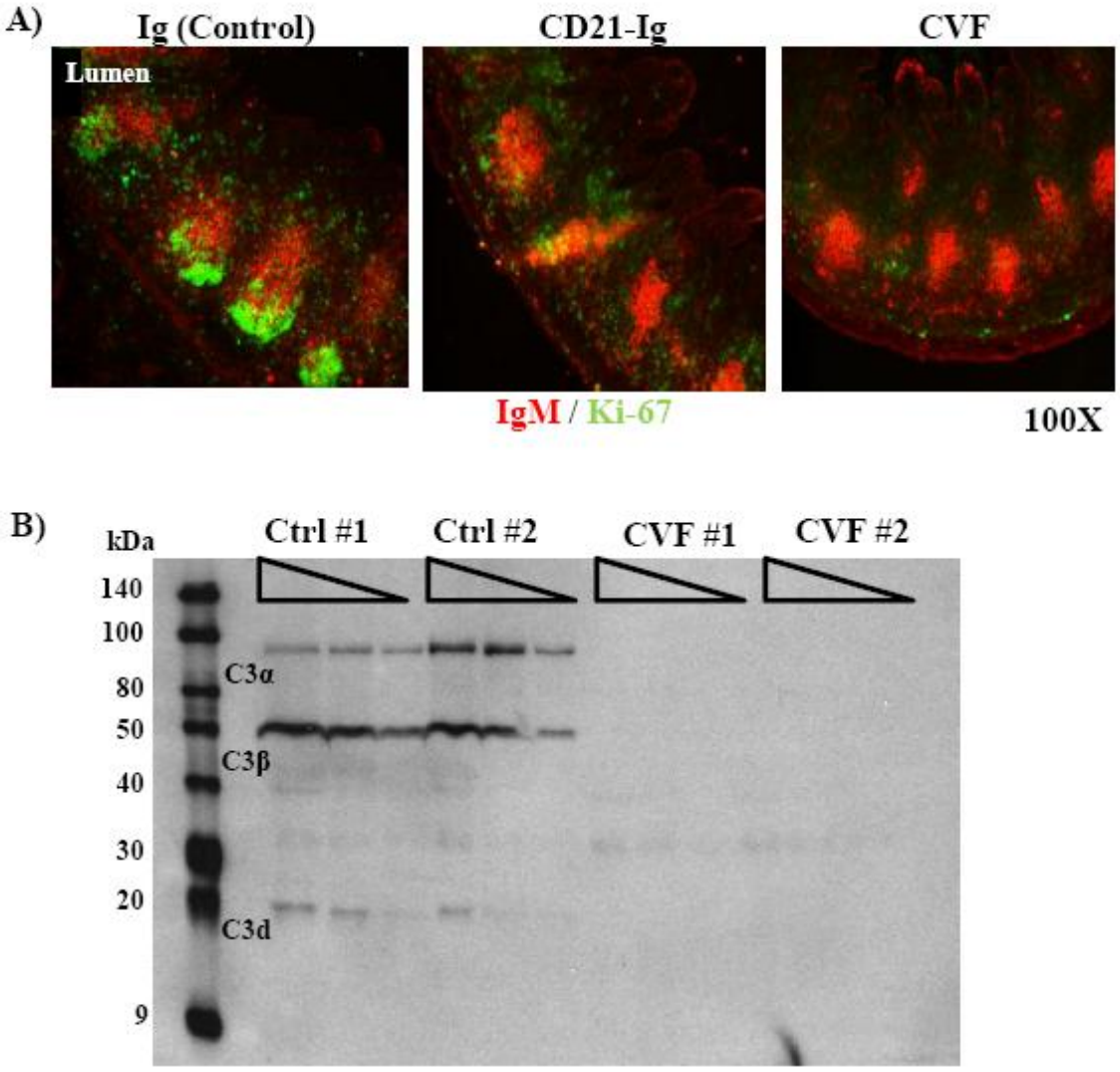


Figure 5: Analysis of B cell proliferation in CD21-Ig-treated, CVF-treated and control (Ig) rabbits. A) Immunofluorescent staining of the appendix for IgM and Ki-67. Data are representative of three Ig (control) and treated (CD21-Ig and CVF) rabbits. Magnification = 100X. B) Western blot of sera from two control PBS-treated (ctrl #1 & #2) and CVF-treated (CVF #1&#2) rabbits, probed with anti-rabbit C3. The open

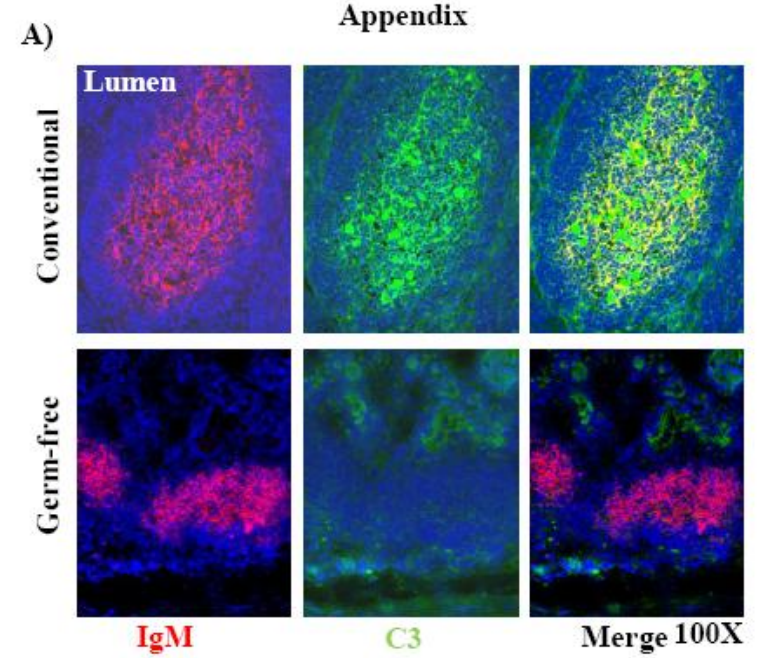
triangles indicate a 2-fold dilution (1, 0.5 and 0.25µl) of the sera across three lanes for each animal.

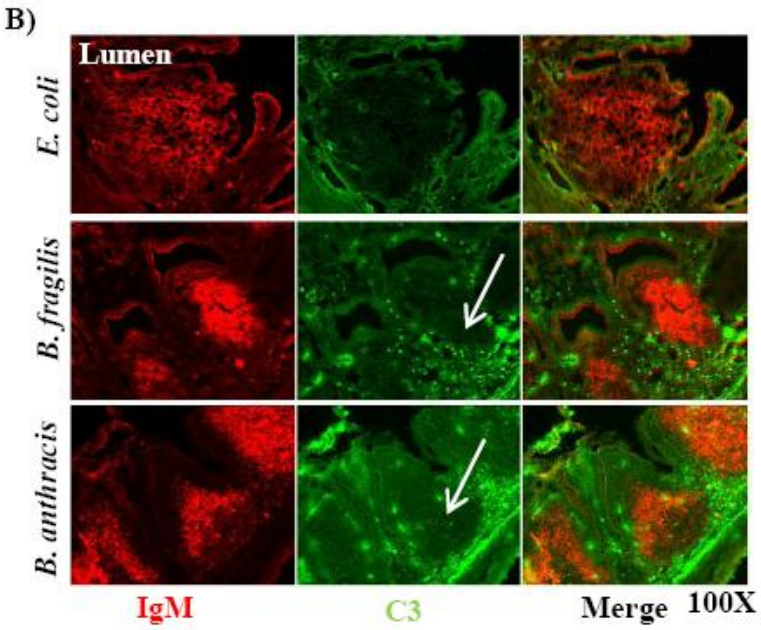
appendices following the introduction of a select group of bacterial species; *E.coli* did not promote B cell expansion, and *B. Fragilis* induced moderate levels of B cell proliferation, while *B. anthracis* induced a relatively robust proliferative expansion of B cells (Lanning et al., 2000a, Rhee et al., 2004, Severson et al., 2010). In these appendices, I found almost no C3 deposition in the follicles when *E.coli* was introduced. In contrast, in the presence of *B. Fragilis* and *B anthracis*, I found staining for C3 in the follicles (Fig 6B), indicating that bacterial species that promote GALT development also induce the expression of complement in GALT. To further characterize the role of complement, I examined if C3 was deposited on the surface of commensal bacteria. By flow cytometry, I found that luminal bacteria were coated with C3 and IgA, and the IgA^+ bacteria appeared to have a greater deposition of C3 on the surface compared to IgA^- bacteria. Taken together, I conclude that commensal bacteria regulate the localization/deposition of complement in GALT.

ROLE OF BAFF AND APRIL DURING B CELL DEVELOPMENT IN GALT

BAFF has been shown to be required for peripheral B cell development in mice, while it is dispensable during B cell development in the primary lymphoid organ, the BM (Schiemann et al., 2001). Because the rabbit appendix is a site for B cell expansion, and considered a primary lymphoid tissue, I examined the role of BAFF and APRIL during the early phase of B cell development in GALT.

Appendix


A)
Lumen
Conventional
Germ-free
IgM
C3
Merge 100X


B)
Lumen
E. coli
B. fragilis
B. anthracis
IgM
C3
Merge 100X

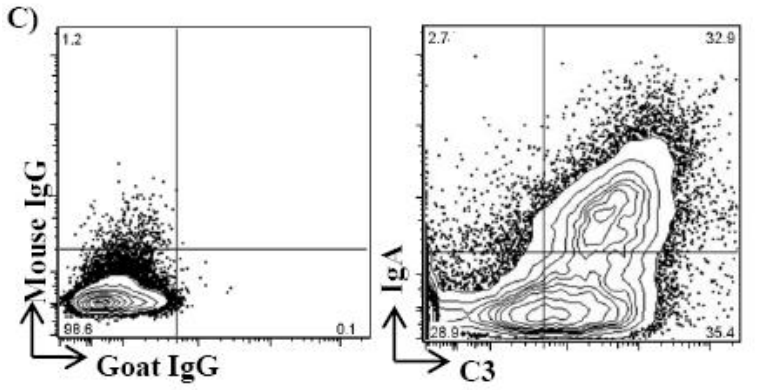
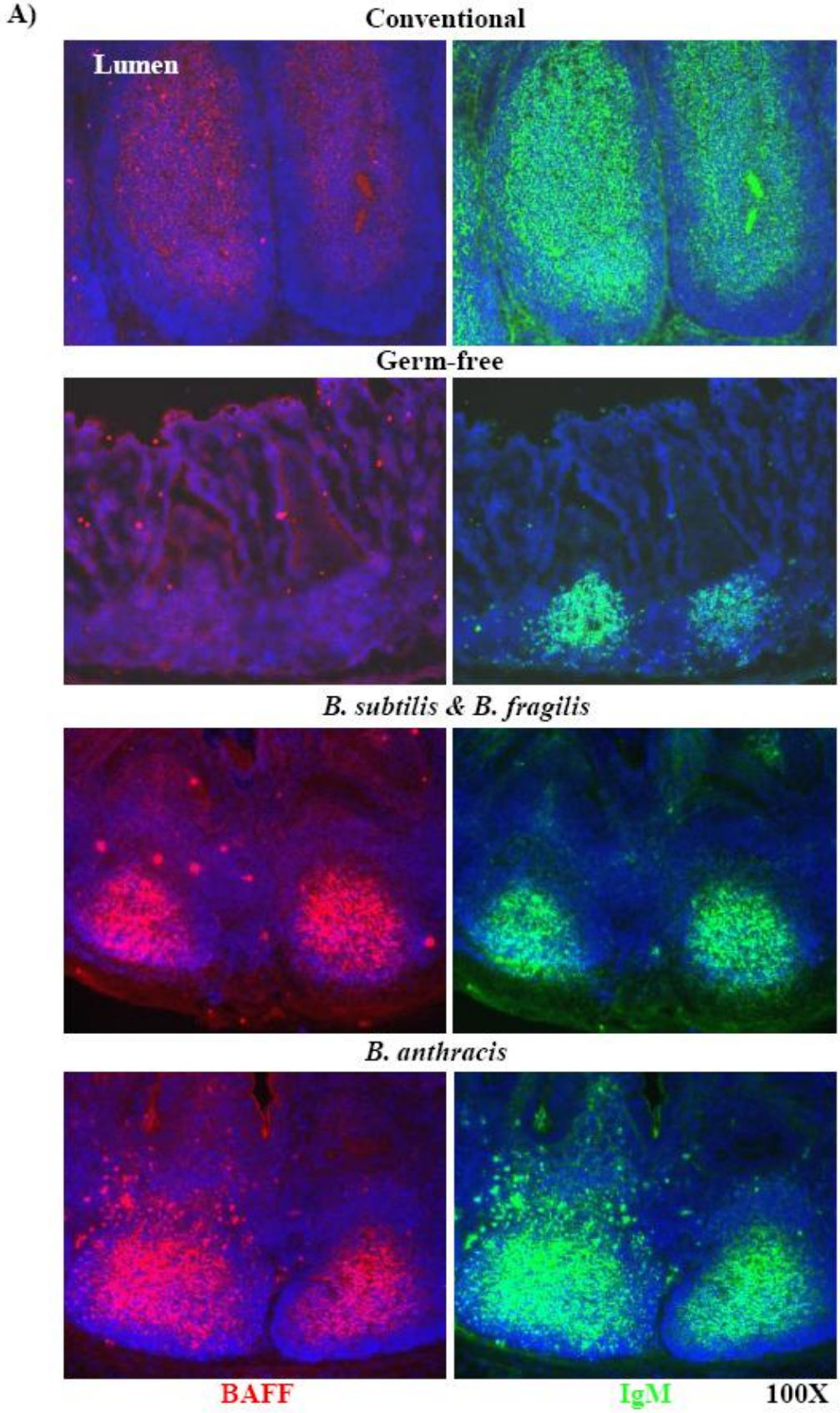

C)
1.2
98.6
0.1
Mouse IgG
Goat IgG
2.7
32.9
28.9
35.4
IgA
C3

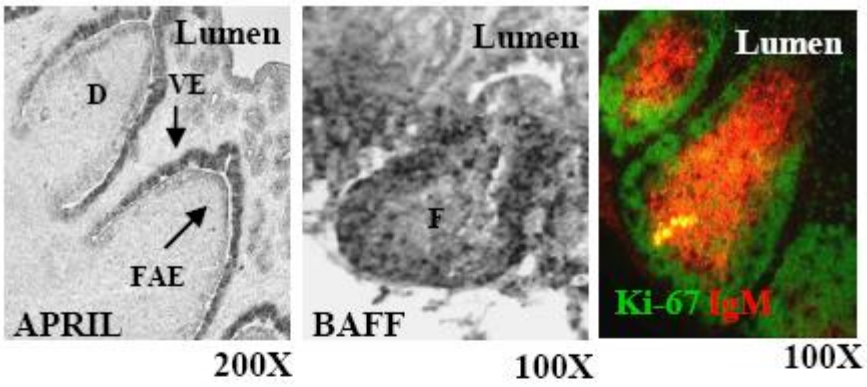
Figure 6: Analysis of appendices from germ-free and conventional rabbits for C3. Immunofluorescent staining for IgM and C3 in appendix sections from conventional 4 wk old rabbit (*upper*) and 4 wk old rabbit with a germ-free appendix (lower) (A) and from GF appendices into which *E.coli* (*upper*), *B.fragilis* (*middle*) and *B.anthracis* (*lower*) were introduced (B). Arrows indicate staining for C3 in the follicles. Magnification = 100X. C) Flow cytometric analysis of IgA- and C3- stained intestinal commensal bacteria from 6 week old rabbit. Plots are representative of two independent experiments. Magnification for all figures = 100X

BAFF and APRIL expression in GALT

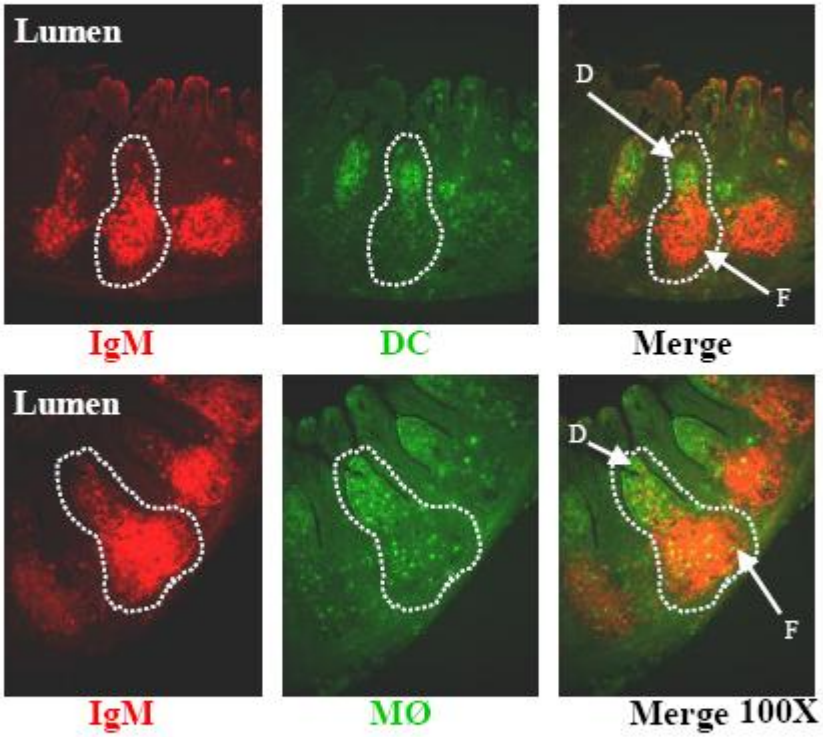
To determine if BAFF and APRIL play a role during B cell development in GALT, I first examined their expression in the appendix, and tested if the expression of BAFF, like complement is regulated by commensal bacteria. I examined BAFF expression in GF and conventional (CV) appendices by immunohistochemistry and found that while BAFF was readily detected in the B cell follicle areas of CV appendices, little to no BAFF was detected in the GF appendices (Fig 7A *upper*). However, following introduction of commensal organisms, *B. subtilis* and *B. fragilis* or *B. anthracis*, BAFF was detected in the B cell follicle areas (Fig 7A *bottom*). These data demonstrate that intestinal microbiota is required for BAFF expression and localization in GALT. By *in situ* hybridization, APRIL transcripts were detected near the FAE and villous epithelium (VE) (Fig 7B *left*). In contrast, BAFF transcripts were detected around the B cell follicles (Fig 7B *middle*), where proliferating B cells reside (Fig 7B *right*). Because BAFF is known to be expressed by macrophages and dendritic cells (DCs) (Schneider et al., 1999, Nardelli et al., 2001), I considered that the BAFF in B cell follicle areas could be derived from resident macrophages and DCs. However, by immunohistochemistry, macrophages and DCs were detected predominantly in the domes (Fig 7C), while most of the BAFF transcripts were detected in the B cell follicles and some in the domes (Fig 7D). These data suggest that the BAFF in B cell follicles is not produced by DCs and macrophages, but instead is produced by B cells.


A)
Conventional
Lumen
Germ-free
B. subtilis & B. fragilis
B. anthracis
BAFF
IgM
100X

B)


Lumen
D
VE
FAE
APRIL
200X
Lumen
F
BAFF
100X
Lumen
Ki-67 IgM
100X

C)


Lumen
IgM
DC
D
F
Merge
Lumen
IgM
MØ
D
F
Merge 100X

D)

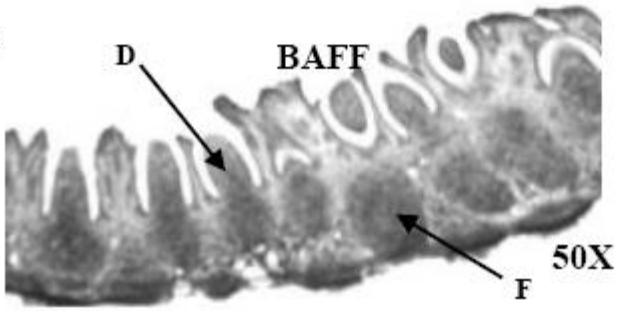

D
BAFF
F
50X

Figure 7: **Immunohistochemistry and *in situ* hybridization to detect BAFF and APRIL in GALT.** A) Immunofluorescent staining for IgM and BAFF in appendix sections from conventional (4 wk old), germ-free (4 wk old), and germ-free appendix rabbits in which the appendix was colonized with *B. subtilis* and *B. fragilis* or *B. anthracis* (7 wk old). B) *In situ* hybridization to detect APRIL (*left*) and BAFF (*middle*) transcripts in appendix (4 week-old CV) and immunofluorescent staining for IgM and Ki-67 (*right*) in appendix (2 week-old CV). C) Immunofluorescent staining of neonatal appendix (1 wk of age) for B cells (anti-IgM) and dendritic cells (DCs) (anti-CD11c) (*top panel*) and macrophages (MΦ) (RAM11) (*bottom panel*). The dotted line indicates a representative follicle (F) and dome (D). D) *In situ* hybridization to detect BAFF transcripts in a neonatal appendix (1 wk-old CV). VE= Villous epithelium; D= dome; F=follicle. Magnification is 100X or 50X.

Expression of BAFF by B cells

To directly test if B cells produce BAFF, I used 55D1 B cells (a rabbit B cell line), and FAC-sorted IgM^+ B cells from appendix, spleen and PB, and performed real time-PCR and western blot analyses. Since myeloid cells express BAFF (Nardelli et al., 2001), I included $CD11b^+$ myeloid cells from the BM and $CD14^+$ monocytes from PB as positive controls. By both assays, I found that 55D1 B cells as well as IgM^+ B cells from appendix, spleen and PB expressed BAFF (Fig 8A and 8B). BAFF, which is a type II transmembrane protein, is proteolytically cleaved on the cell surface to generate soluble BAFF (sBAFF) (Schneider et al., 1999). To determine if BAFF expression can be detected on the surface of B cells, I stained primary B cells and two B cell lines, 55D1 and PBL-1 (Sethupathi et al., 1994, Knight et al., 1988), with biotinylated TACI-Ig. Some of the B cells bound TACI-Ig (Fig 8C), albeit weakly, indicating that they express BAFF. As a positive control, CHO cells transfected with full length rabbit BAFF readily bound TACI-Ig (Fig 8D). Together, these results show that rabbit B cells express BAFF. Recent reports demonstrated BAFF transcript and protein expression in mouse and human B cells; however, membrane expression of BAFF was undetectable on most freshly isolated human peripheral B cells, presumably, due to rapid shedding of BAFF (Darce et al., 2007, Li et al., 2008). In mice, only B1 B cells displayed low levels of membrane BAFF (Chu et al., 2007). Thus, in rabbits, I suggest that the BAFF expressing B cells may represent a B1-like subset.

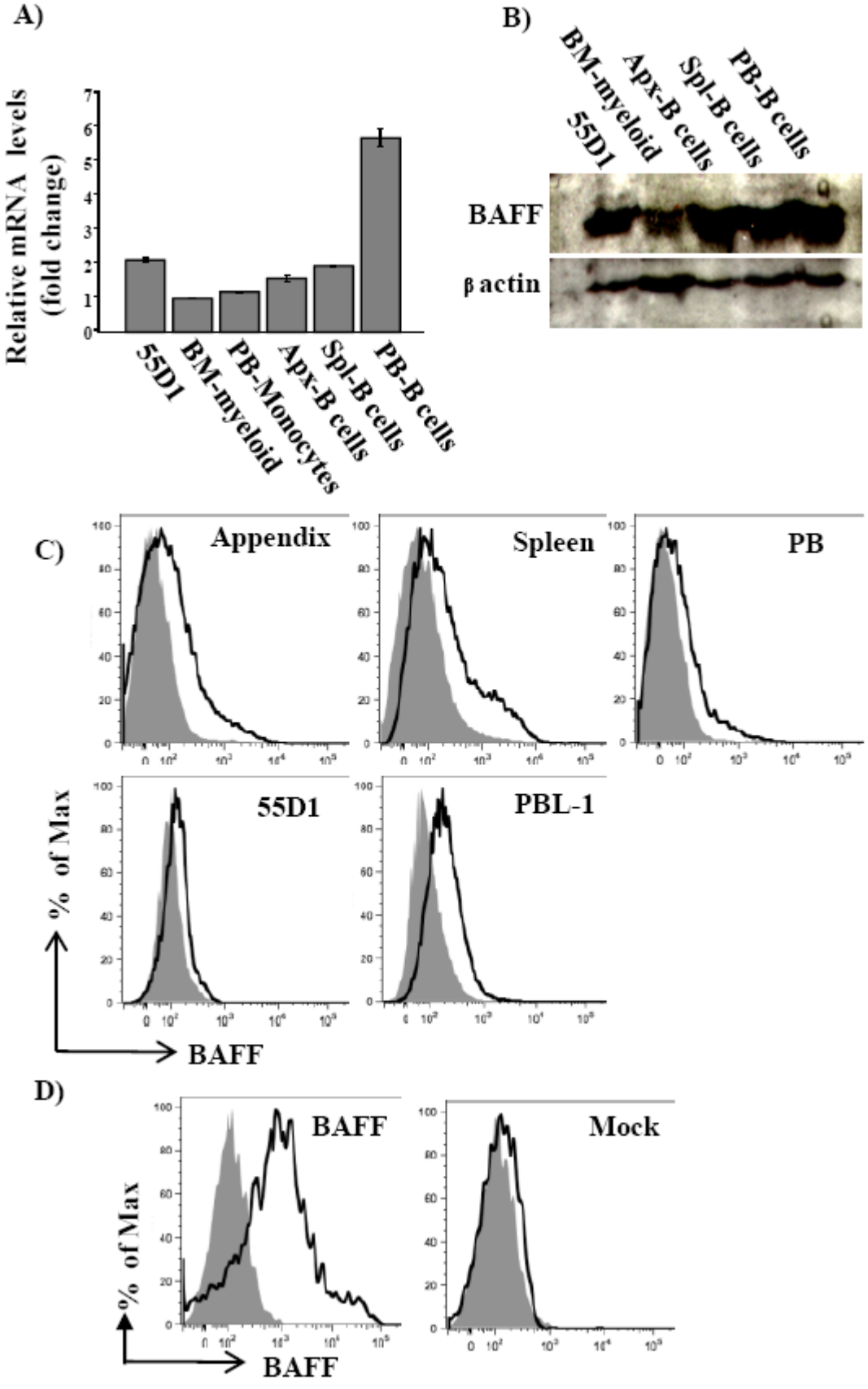

A)
Relative mRNA levels (fold change)
55D1
BM-myeloid
PB-Monocytes
Apx-B cells
Spl-B cells
PB-B cells
B)
55D1
BM-myeloid
Apx-B cells
Spl-B cells
PB-B cells
BAFF
β actin
C)
Appendix
Spleen
PB
55D1
PBL-1
% of Max
BAFF
D)
BAFF
Mock
% of Max
BAFF

Figure 8: BAFF expression in B cells. A) Quantitative-PCR for BAFF transcripts from 55D1 B cells, BM CD11b$^+$ myeloid cells, PB CD14$^+$ monocytes, and IgM$^+$ appendix (Apx), spleen (Spl), PB B cells. Data are represented as fold changes of BAFF mRNA relative to BM CD11b$^+$ myeloid cells. Error bars= standard error of the mean derived from triplicate PCR reactions. B) Western blot of lysates from 55D1 cells, BM CD11b$^+$ myeloid cells and IgM$^+$ Apx, Spl and PB B cells probed with anti-BAFF. C) Flow cytometric analysis of IgM$^+$ cells from appendix, spleen, PB, and B cell lines, 55D1 and PBL-1, stained with biotinylated TACI-Ig (open histograms) and human IgG as isotype control (shaded histograms). D) Flow cytometric analysis of CHO cells transfected with full length BAFF and stained with biotinylated TACI-Ig (open histogram) or biotinylated human IgG as isotype control (shaded histogram). Mock = CHO cells transfected with empty vector. Data are representative of two (qPCR), four (western blot) and three (flow cytometry) experiments.

Requirement of BAFF/APRIL for GALT development

To determine if BAFF and/or APRIL are required for B cell expansion in GALT, I used a soluble decoy receptor approach to neutralize these cytokines *in vivo*. I injected newborn rabbits with rAd expressing TACI-Ig, and after 7-10 days analyzed the appendix by immunohistochemistry for the presence of Ki-67^+ proliferating B cells (Fig 9A). I found little to no Ki-67 expression in the appendix, and the B cell follicles were markedly reduced in size compared to control rabbits. Consistent with these immunohistochemical results, by flow cytometry, I found the percentage of IgM^+ B cells was greatly reduced in TACI-Ig treated rabbits, when compared to Ig controls (16.8% vs. 47%) (Fig 9B). Similar to the appendix, B cells in the spleen and PB were markedly reduced (Fig 9A *lower* and 9B). In BM, I did not find any perturbation in the B cell compartment of TACI-Ig treated rabbits (Fig 9C). The frequency of precursor B cell populations, pro and preB cells, as well as antigen presenting cells ($MHCII^+$) and other hematopoietic ($CD43^+$) precursors were similar between rabbits injected with TACI-Ig and control rAd. Taken together, these experiments indicate that neutralization of BAFF/APRIL by TACI-Ig blocked B cell development in GALT and peripheral tissues, while leaving the BM B cell compartment intact.

Stimulation of appendix B cells by BAFF and APRIL

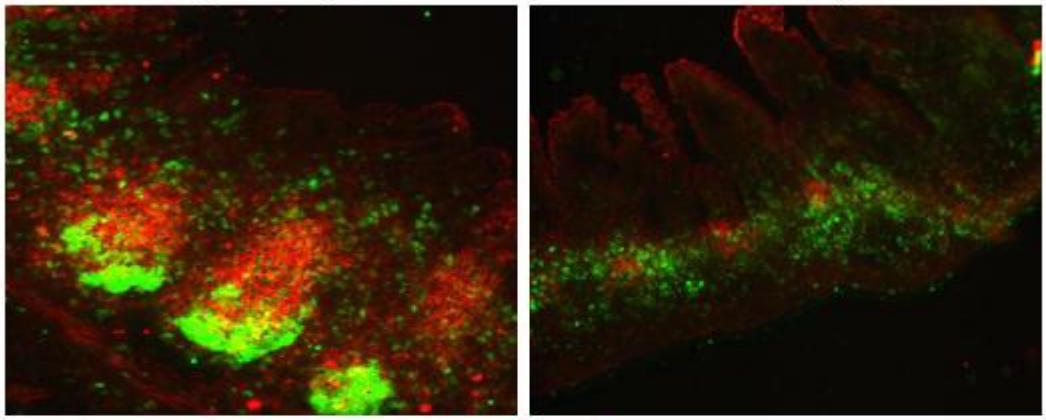
Inhibition of B cell development in GALT by the BAFF/APRIL decoy receptor suggested to me that appendix B cells may be stimulated to proliferate by these

A)

Appendix

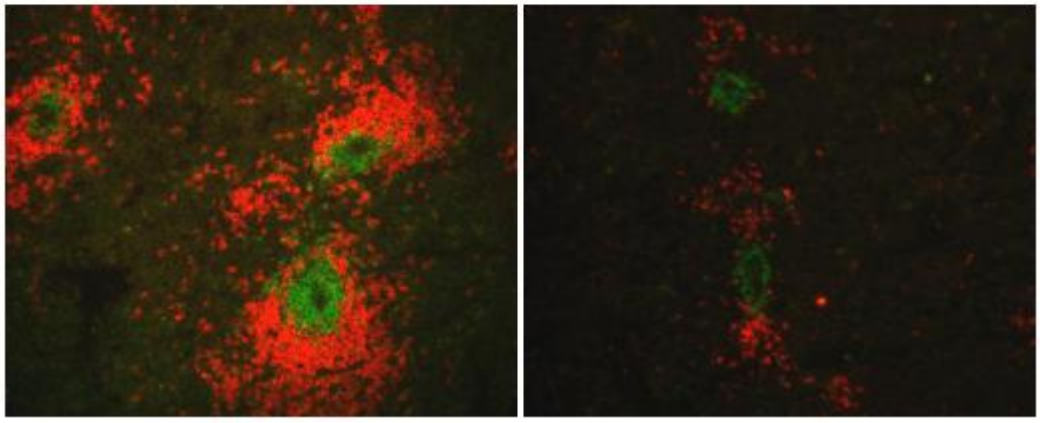
Ig
(Control)

TACI-Ig



IgM / Ki-67

Spleen



IgM / CD4

100X

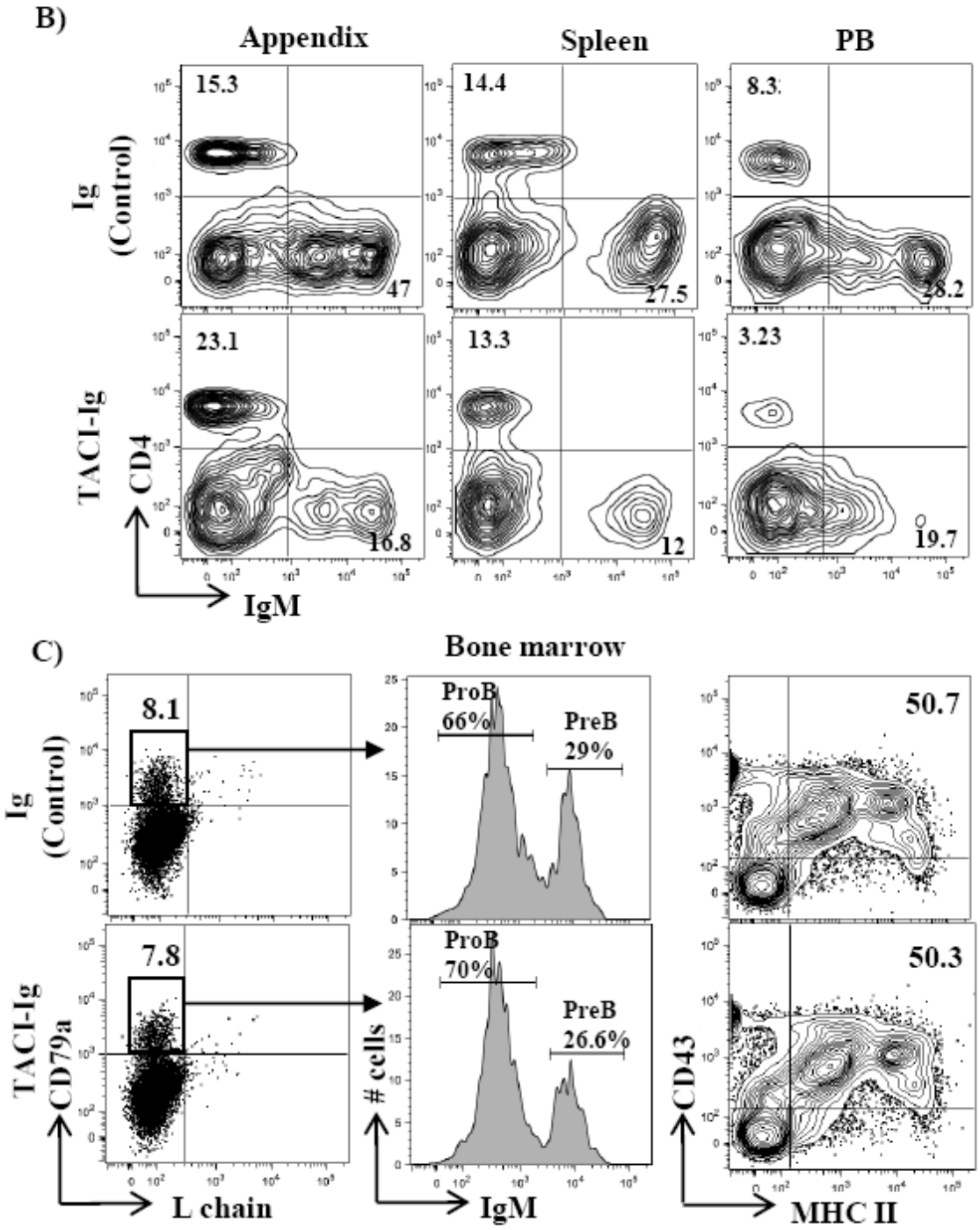

B)
Appendix
Spleen
PB
Ig (Control)
15.3
47
14.4
27.5
8.3
28.2
TACI-Ig
CD4
23.1
16.8
13.3
12
3.23
19.7
IgM
C)
Bone marrow
Ig (Control)
8.1
ProB 66%
PreB 29%
50.7
TACI-Ig
CD79a
7.8
ProB 70%
PreB 26.6%
50.3
cells
L chain
IgM
CD43
MHC II

Figure 9: Analysis of the B cell compartment in TACI-Ig-treated and control (Ig) rabbits. A) Immunofluorescent staining for IgM and Ki-67 in the appendix and for IgM and CD4 in spleen B) Flow cytometry of IgM and CD4 stained cells in appendix, spleen and PB. C) Flow cytometry of bone marrow stained with antibodies to surface L chain and cytoplasmic CD79a (left), cytoplasmic IgM (center) and CD43 and MHC II (right). Data are representative of three control rabbits and five TACI-Ig treated rabbits.

cytokines. To test if BAFF and APRIL co-stimulate GALT B cells, I cultured appendix cells with anti-Ig and recombinant soluble BAFF (rBAFF) and APRIL (rAPRIL) and measured proliferation by [^{3}H]-thymidine incorporation. While splenocytes exhibited a robust dose-dependent proliferative response to both rBAFF and rAPRIL, appendix B cells showed only a modest two-fold increase in [^{3}H]-thymidine incorporation (Fig 10A and 10B). As a positive control for proliferation, appendix B cells readily responded to CD40L stimulation (Fig 10C). To investigate whether BAFF provides a survival signal to GALT B cells, I cultured appendix B cells with BAFF and found approximately two to four-fold more viable B cells in rBAFF-containing cultures as compared to media alone, or denatured BAFF containing cultures (Fig 10D). I conclude that neither BAFF nor APRIL provide a strong co-stimulatory (proliferative) signal to appendix B cells. Appendix B cells also did not proliferate in response to anti-Ig stimulation alone, indicating that B cells in appendix are functionally different from those in spleen.

Occupancy of BAFF binding receptor(s) on primary B cells

To determine the expression pattern of BAFF-binding receptors(s) (BBRs) on GALT and peripheral B cells, I stained B cells with biotinylated rBAFF and was surprised to find that most B cells from appendix and PB did not bind rBAFF (Fig 11A). This was unexpected because studies with primary human and murine B cells showed rBAFF binding to almost all B cells (Avery et al., 2003, He et al., 2004, Schneider et al., 1999, Thompson et al., 2001). I ruled out the possibility that the lack of binding was due to defective rBAFF reagent by testing it on B cell lines and found that rBAFF bound to

five of six B cell lines of rabbit, human and mouse origin (Fig 11B). Next, I considered that the lack of binding of rBAFF to primary B cells could be due to the lack of BR3, a predominant receptor on murine and human B cells. However, by flow cytometry, I found B cells from appendix, PB, and spleen expressed BR3 (Fig 12A). To test if the lack of rBAFF binding was due to occupied receptors, I used an anti-BAFF antibody that primarily detects surface bound BAFF (Darce et al., 2007) and found that all B cells reacted with this antibody (Fig 12B), suggesting that the BBRs have endogenous BAFF already bound and therefore are unavailable to bind rBAFF. However, primary B cells that were activated *in vitro* with anti-Ig and CD40L did bind rBAFF (Fig 12C), suggesting that upon activation, B cells either lose surface bound BAFF and/or synthesize new BBRs that can bind rBAFF. I conclude that in rabbits, most primary B cells do not bind rBAFF because the BBRs are already occupied.

Binding of rBAFF to peripheral blood monocytes and splenic marginal zone B cells

As shown in Fig 11A, I observed some cells in PB and spleen that bound rBAFF. Surprisingly, most of these rBAFF-binding cells in blood were $MHCII^{+}$ (Fig13A), $CD11b^{+}$ and $CD14^{+}$ (Fig 13B), indicating that BAFF binds PB monocytes. Ritter et al. (2006) showed that CD44 could be used as a marker to distinguish between murine myeloid and lymphoid cells, and I first used anti-CD44 mAb to determine if CD44 could distinguish rabbit myeloid and lymphoid cells. I identified $CD44^{lo}$ and $CD44^{hi}$ subsets of $MHCII^{+}$ cells in PB (Fig 13C *upper*) and showed that the $CD44^{hi}$ subset consisted of mostly $CD14^{+}$ and $CD9^{+}$ myeloid cells (Fig 13C *lower middle* and *right*) and the $CD44^{lo}$

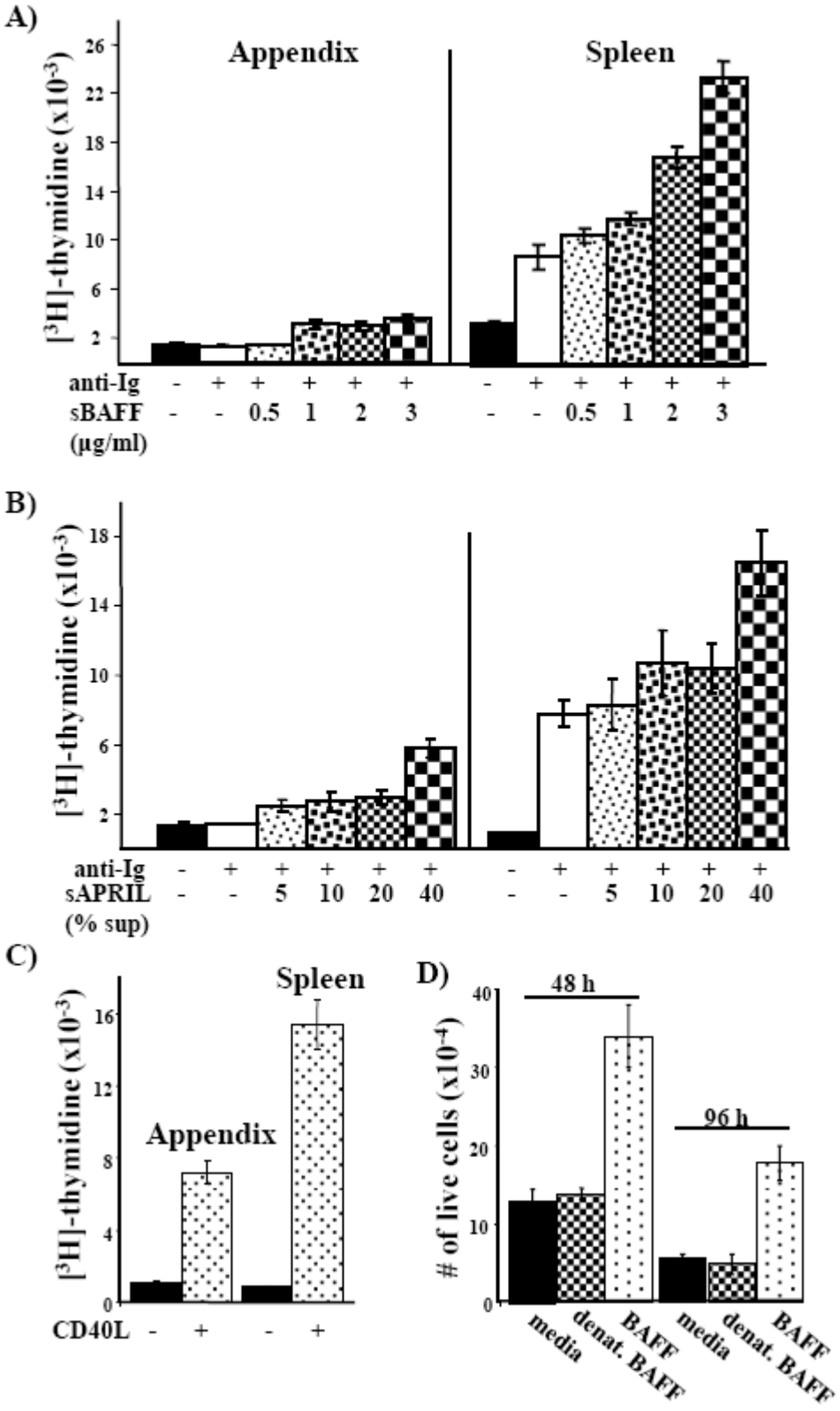

A)
Appendix
Spleen
[3H]-thymidine (x10-3)
anti-Ig
sBAFF (µg/ml)
B)
[3H]-thymidine (x10-3)
anti-Ig
sAPRIL (% sup)
C)
Spleen
Appendix
[3H]-thymidine (x10-3)
CD40L
D)
48 h
96 h
# of live cells (x10-4)
media
denat. BAFF
BAFF

Figure 10: *In vitro* stimulation of GALT B cells with recombinant BAFF and APRIL. Splenocytes and appendix cells cultured for 72 hrs with anti-Ig (10µg/ml) and increasing concentrations of A) soluble BAFF (sBAFF) or B) supernatant containing soluble APRIL (sAPRIL) (5-40%). Proliferation was assessed by ^{3}[H]-thymidine incorporation. C) Splenocytes and appendix B cells co-cultured with and without CD40L-expressing CHO cells in a 100:1 ratio, respectively. Proliferation was assessed by ^{3}[H]-thymidine incorporation. D) $CD4^{+}$ depleted appendix B cells were cultured in either medium alone, or in media supplemented with sBAFF or denatured sBAFF. Live cells in culture were enumerated after 48 and 96 hours (h). Error bars = standard error of the mean derived from triplicate wells. Data are representative of three independent experiments.

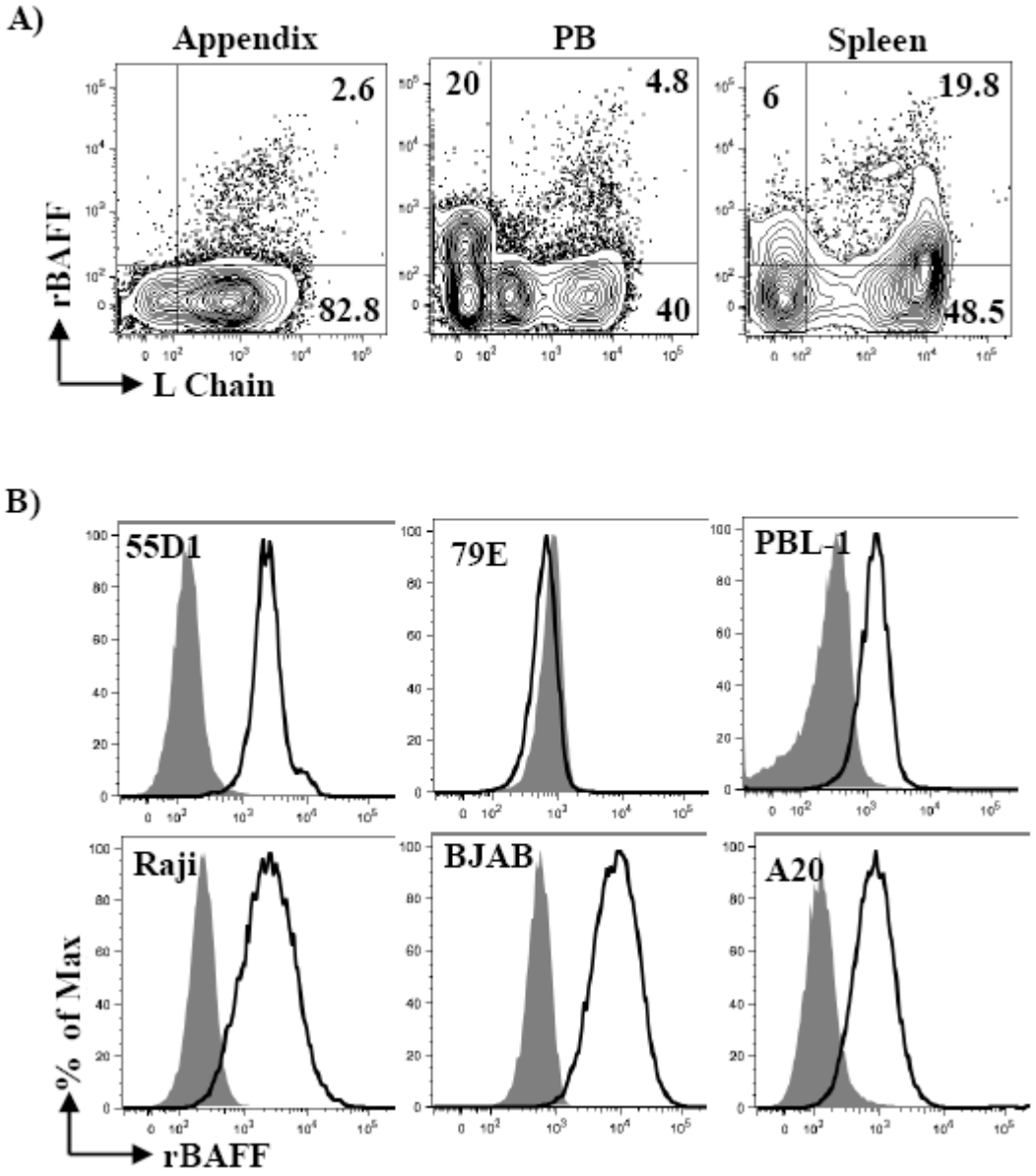


Figure 11: Flow cytometric analysis of rBAFF-binding to B cells. A) Staining of freshly isolated appendix, PB and spleen cells with anti-L chain and biotinylated rBAFF. B) Histograms of B cell lines, 55D1, 79E and PBL-1 (rabbit), Raji and BJAB (human) and A20 cells (mouse) stained with biotinylated rBAFF (open histograms) followed by

streptavidin PE. Negative control= cells stained with steptavidin PE only (shaded histograms).

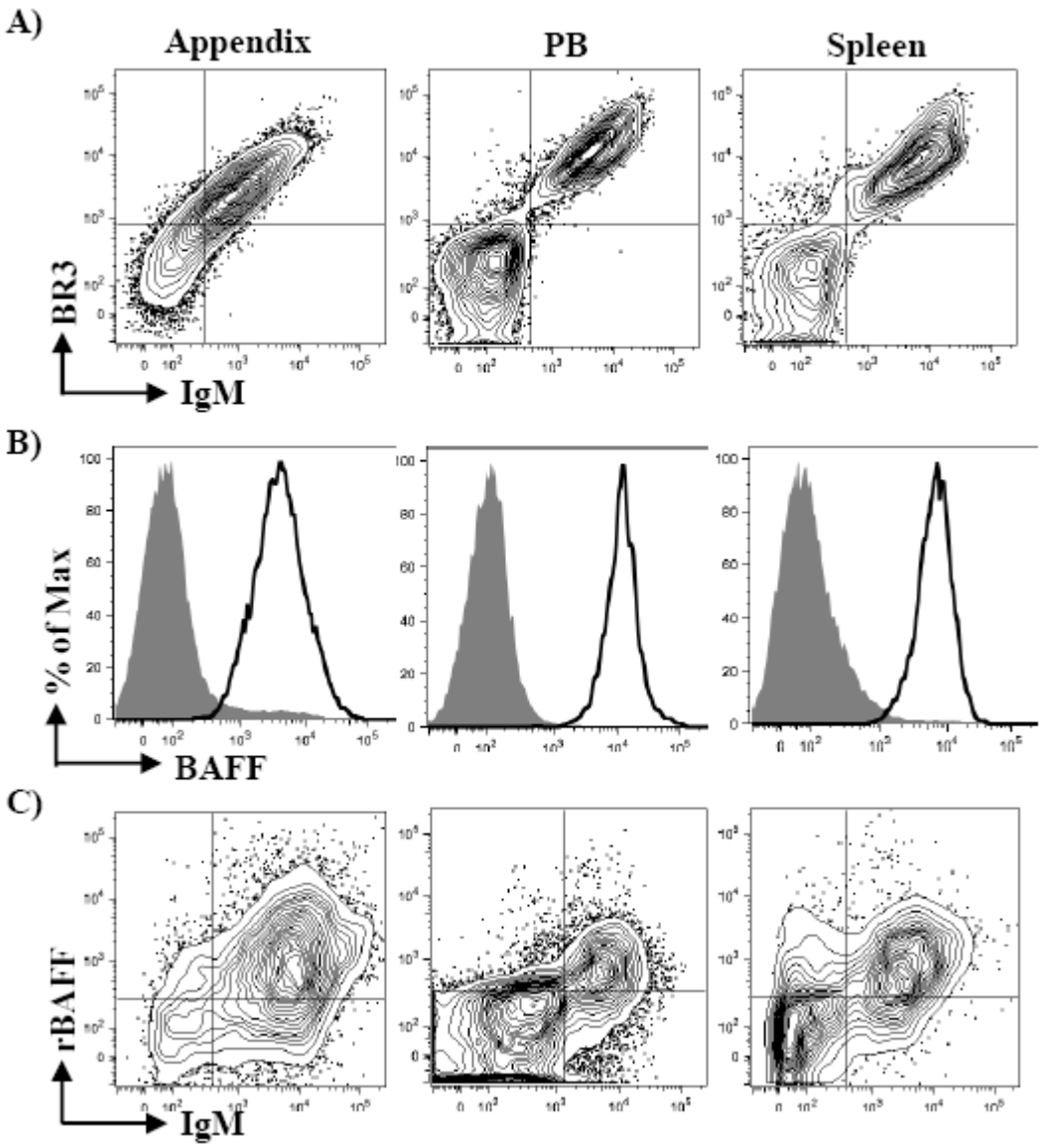


Figure 12: Flow cytometric detection of BR3 and BAFF on appendix, PB and splenic B cells. Cells stained with A) anti-IgM and goat anti-BR3 followed by biotinylated rabbit (Fab) anti-goat Ig and streptavidin PE. B) Gated IgM^+ cells stained with goat anti-BAFF

followed by biotinylated rabbit anti-goat Ig and streptavidin PE. C) Anti-Ig and CD40L activated B cells stained with anti-IgM and rBAFF.

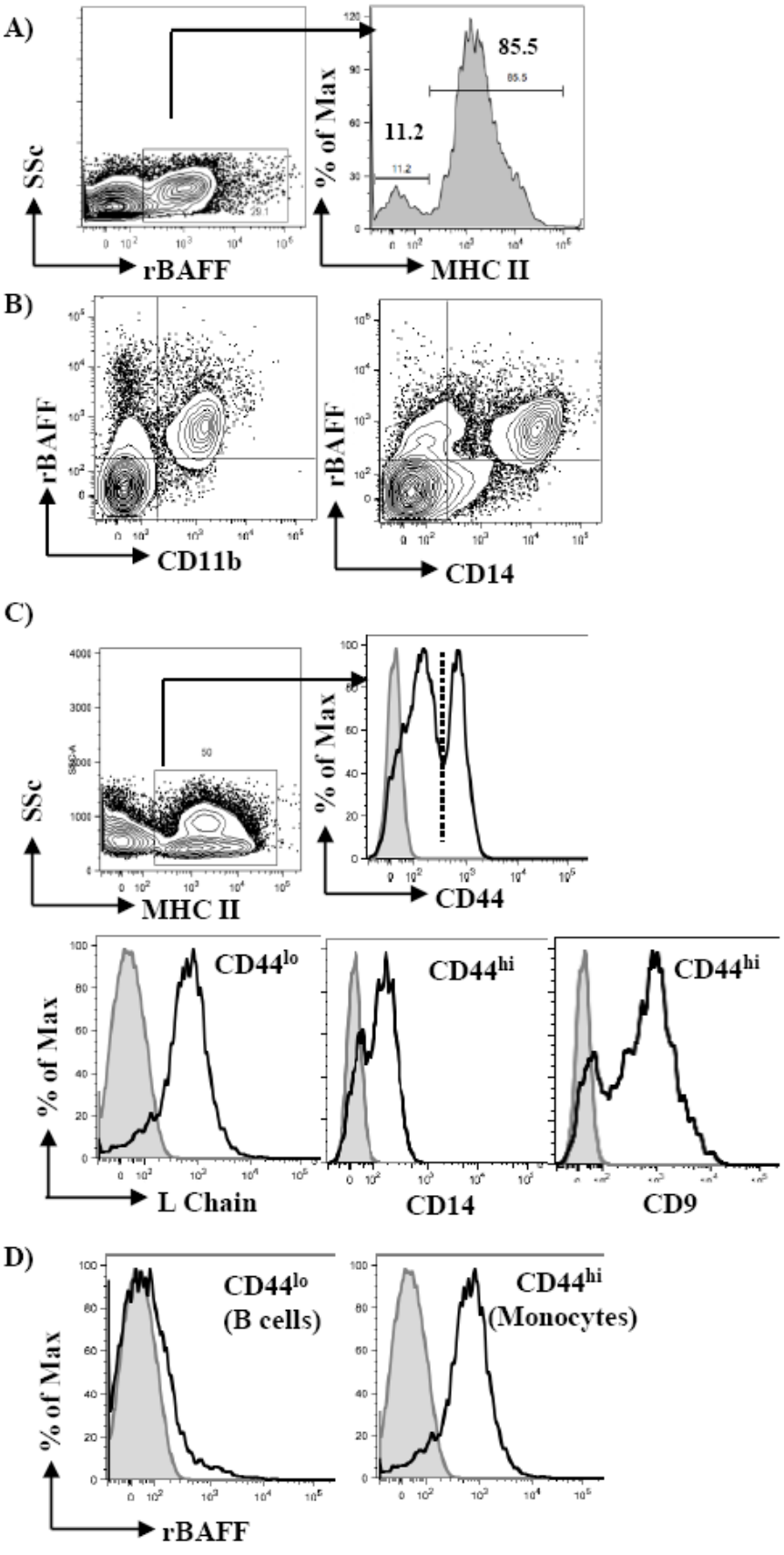

A)
SSc
rBAFF
% of Max
85.5
11.2
MHC II
B)
rBAFF
CD11b
rBAFF
CD14
C)
SSc
MHC II
% of Max
CD44
% of Max
CD44lo
L Chain
CD44hi
CD14
CD44hi
CD9
D)
% of Max
CD44lo
(B cells)
rBAFF
CD44hi
(Monocytes)

Figure 13: BAFF-binding to PB monocytes. Flow cytometric staining of PB lymphocytes with A) rBAFF and anti-MHCII, B) anti-CD11b, anti-CD14 and rBAFF. C) *Top*: Flow cytometric analysis of $MHCII^{+}$ cells for CD44 expression. $CD44^{hi}$ and $CD44^{lo}$ subsets indicated by vertical dashed line. *Bottom*: Analysis of $CD44^{lo}$ cells for L chain expression (*left*) and $CD44^{hi}$ cells for CD14 and CD9 expression (*middle* and *right*). D) Flow cytometric staining of $CD44^{hi}$ and $CD44^{lo}$ subsets of MHC II^{+} cells with rBAFF. Shaded histogram= isotype control.

subset comprised of L chain^{+} B cells (Fig 13C *lower left*). By using CD44 as a pan marker to distinguish rabbit myeloid and lymphoid cells (Fig 13D), I confirmed that rBAFF binds strongly to CD44^{hi} myeloid cells and only weakly to CD44^{lo} B cells. In the spleen, most of the BAFF-binding cells were B cells (Fig 11A). Since *in vitro* activated B cells bound rBAFF (Fig 12C) and MZ B cells are likely activated and express high levels of TACI (Kanswal et al., 2008), I tested if the subset of splenic B cells that bound rBAFF were MZ B cells. Because CD9 is expressed on some MZ B cells in mice (Won and Kearney, 2002), I used anti-rabbit CD9 mAb to identify MZ B cells in rabbit spleen. By immunohistochemistry, I found BAFF- binding and CD9 expression around the margins of B cell follicles (Fig 14A), suggesting that BAFF may be binding to MZ B cells. By flow cytometry, using anti-CD9 mAb, rBAFF and anti-L chain, I found that a subset of CD9^{+} B cells bound rBAFF (Fig 14B). Taken together, I conclude that rBAFF binds PB monocytes and also to a subset of CD9^{+} splenic MZ B cells.

Binding of rBAFF to a subset of IgM^{lo} B cells

Although most freshly isolated B cells from adult rabbit PB and appendix did not bind rBAFF (Fig 11A), in neonates, I found a subset of IgM^{lo} B cells from appendix and spleen that did bind rBAFF (Fig15A *left panel*). In the appendix follicles, these rBAFF^{+} cells were located in the basolateral region (Fig 15B) where Ki-67^{+} proliferating B cells reside (Fig 9A), suggesting that these subsets are cycling *in vivo*. Further, in TACI-Ig treated rabbits, over 30% of the B cells in appendix (11.6% of 30.2%) were IgM^{lo} and bound rBAFF, while only about 10% of the B cells in the control rabbits (6.5% of 70.3%)

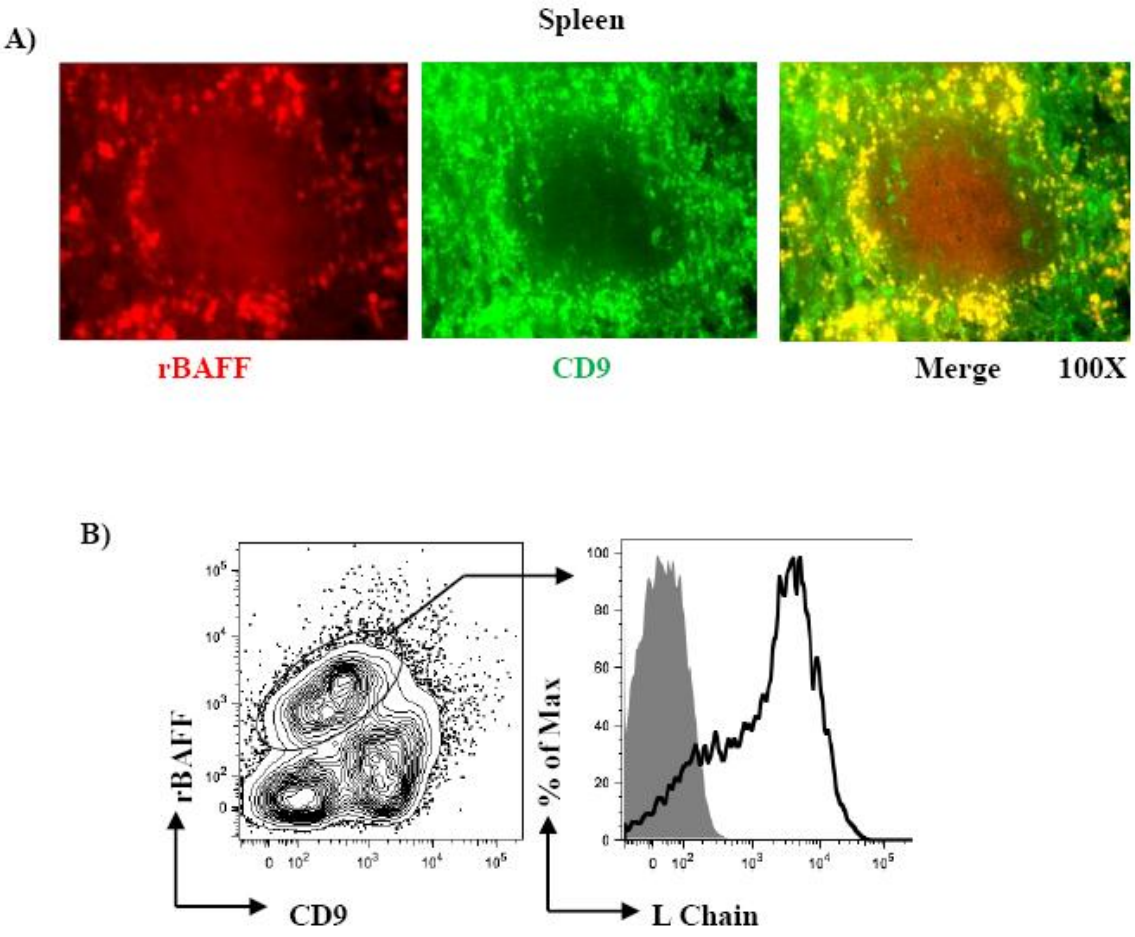
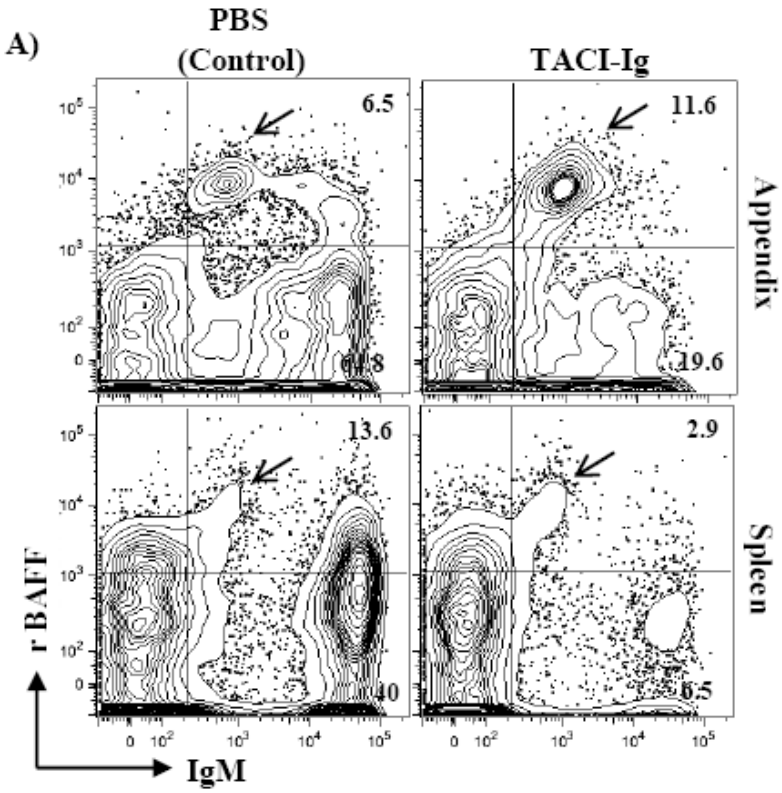
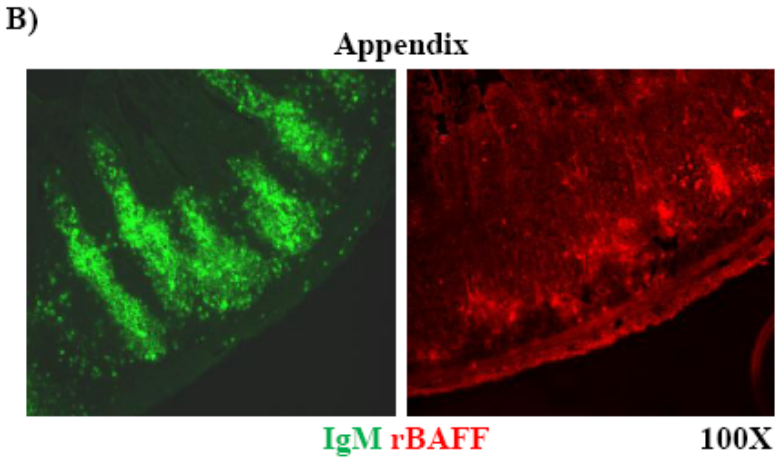


Figure 14: BAFF-binding to splenic MZ B cells. A) Immunofluorescent staining of spleen with rBAFF and anti-CD9 mAb. B) Flow cytometric analysis of splenocytes with anti-CD9, rBAFF and anti-L chain.


A)
PBS
(Control)
TACI-Ig
6.5
11.6
Appendix
64.8
19.6
13.6
2.9
Spleen
40
6.5
r BAFF
IgM


B)
Appendix
IgM rBAFF
100X

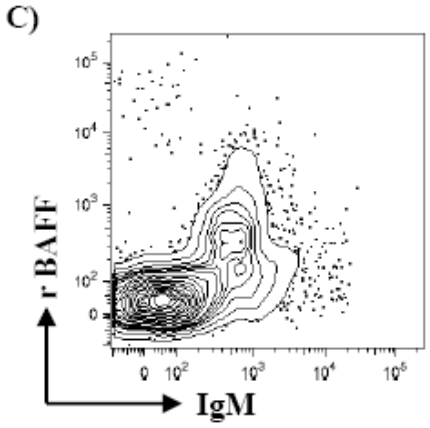

C)
r BAFF
IgM

Figure 15: Identification of an IgM^{lo} BAFF-binding B cell subset (transitional-like B cell) in neonates. A) Staining of appendix and spleen cells from TACI-Ig treated or control rabbits with anti-IgM and rBAFF. Arrow identifies IgM^{lo} rBAFF-binding B cells. B) Immunofluorescent staining of normal appendix with anti-IgM and rBAFF. C) Staining of PB from a newborn rabbit with anti-IgM and rBAFF. Data in A) are representative of two rabbits in each group.

bound rBAFF (Fig 15A). This accumulation of rBAFF^{+} IgM^{lo} B cells in TACI-Ig treated rabbits suggests that this subset depends on BAFF for maturation and represents transitional-like B cells that arise early in development. In support of this idea, I found that most B cells in the peripheral blood of a newborn rabbit (2 day old), expected to be immature or transitional-like B cells were IgM^{lo} and many of these bound rBAFF (Fig 15C). In the next section, I further characterized these transitional-like B cells.

IDENTIFICATION OF TRANSITIONAL B CELLS

The presence of a rBAFF-binding IgM^{lo} transitional-like B cell subset in neonates, and their accumulation in TACI-Ig treated rabbits, suggested to me that B cells in rabbit go through a transitional-like stage during development. To understand the biology of these B cells, I further phenotypically and functionally characterized them in young and adult rabbits.

Identification of transitional B cell subsets

One major difficulty in identifying B cell subpopulations in rabbits is the lack of antibody reagents. As I mentioned previously, the only B cell markers available are antibodies to IgM or cytoplasmic CD79a which are pan B cell markers and would not allow me to easily identify B cell subsets. To overcome this difficulty, I discovered several crossreactive antibodies and identified B cell subpopulations in adult rabbits, based on the expression of surface markers used to delineate immature and mature B cells in mice and humans. Because human CD24 and its murine homologue, HSA, are

expressed early in B cell development on both BM B cell progenitors and transitional B cells, but are downregulated on mature B cells (Allman et al., 1992, Carsetti et al., 2004), I tested if anti-CD24 can be used to identify transitional B cells in rabbits. Using anti-CD21 and anti-CD24 mAbs, I identified two subsets of $CD24^{hi}$ B cells in the spleen ($CD21^{lo}$ and $CD21^{+}$), which I will henceforth refer to as T1 and T2 B cells, respectively, and a $CD24^{-}CD21^{+}$ subset, designated mature (M) B cells (Fig 16A *top*). T1 B cells were IgM^{lo} $CD62L^{lo}$, while both T2 and mature B cells had higher levels of CD21, surface IgM, and CD62L expression (Fig 16A *top*). CD23 was expressed at similar levels on both T1 and T2 B cells (Fig 16A *top*) and thus did not serve as a useful marker to distinguish between these B cell subsets. To determine if these transitional B cells share features with human transitional B cells, which are broadly defined as $CD24^{hi}CD38^{hi}CD10^{+}CD20^{hi}$ (Cuss et al., 2006), I analyzed the $CD24^{hi}$ cells for these markers and found that the T1 B cells were $CD10^{lo}CD38^{+}$, while the T2 B cells were $CD10^{hi}$ and $CD38^{hi}$ (Fig 16A *bottom*). Interestingly, CD20 was expressed on the T1 and T2 cells but not on the mature B cells, and thus served as a unique marker to identify transitional B cells (Fig 16A *bottom*). Further, T1 and T2 B cells expressed high levels of CD90 (Fig 16A *bottom*), a phenotype shared with rat immature B cells (Dammers et al., 2000). Unlike in spleen, I observed only a single subset of $CD24^{hi}$ cells in the PB and these had a lower expression of CD21 compared to mature B cells (Fig 16B *left*). These cells were $IgM^{lo}CD62L^{lo}$ (Fig 16B *right*), suggesting that PB contains only a T1-like population of transitional B cells. I observed a similar T1-like $CD21^{lo}CD24^{hi}$ subset in GALT [appendix (Apx), sacculus rotundus (SR), Peyer's patch (PP) and mesenteric lymph node (MLN)] (Fig 16C). Taken

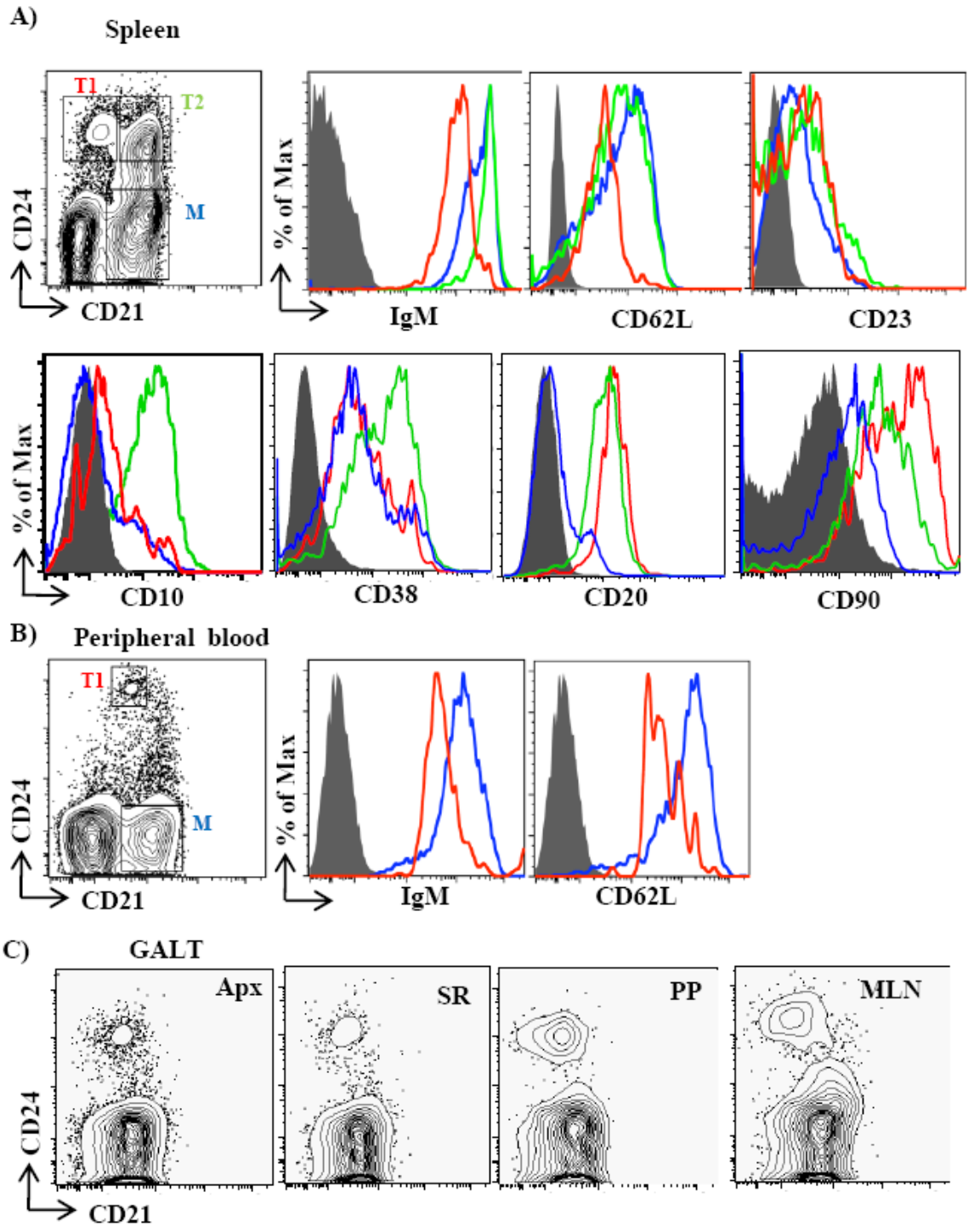

A)
Spleen
T1
T2
M
CD24
CD21
% of Max
IgM
CD62L
CD23
CD10
CD38
CD20
CD90
B)
Peripheral blood
T1
M
CD24
CD21
% of Max
IgM
CD62L
C)
GALT
Apx
SR
PP
MLN
CD24
CD21

Figure 16: Flow cytometric identification of transitional B cell subsets in adult rabbits. A) Staining of splenic B cells for CD21 and CD24, with IgM, CD62L, CD23, CD10, CD38, CD20 and CD90 histograms of cells in T1 (red), T2 (green) and mature (M) (blue) cell gates. B) Staining of PB lymphocytes for CD24 and CD21, with IgM and CD62L histograms of cells in T1 (red) and M (blue) cell gates. C) Lymphocytes from appendix (Apx), sacculus rotundus (SR), Peyer's patches (PP) and mesenteric lymph nodes (MLN) stained for CD24, CD21 and IgM; analysis of cells in IgM^+ gate is shown. The gray filled histograms in A and B represent staining with an appropriate isotype control mAb. The plots are representative of staining obtained from at least 3 rabbits.

Table 3: Frequency of transitional B cells in adult rabbits

Tissue[a]	**T1**[b]	**T2**[b]
Spleen (11)	7.2 ± 2	13.6 ± 2.3
Blood (6)	2.9 ± 0.9	ND
Appendix (6)	2 ± 0.4	ND
Sacculus rotundus (3)	3.8 ± 0.9	ND
Mesentric lymph node (4)	3.7 ± 0.9	ND
Peyer's patch (2)	9.8 ± 3.8	ND

[a]The number in parentheses indicates the number of rabbits analyzed

[b]Indicates the percent of total B cells and ± represents the standard error of the mean

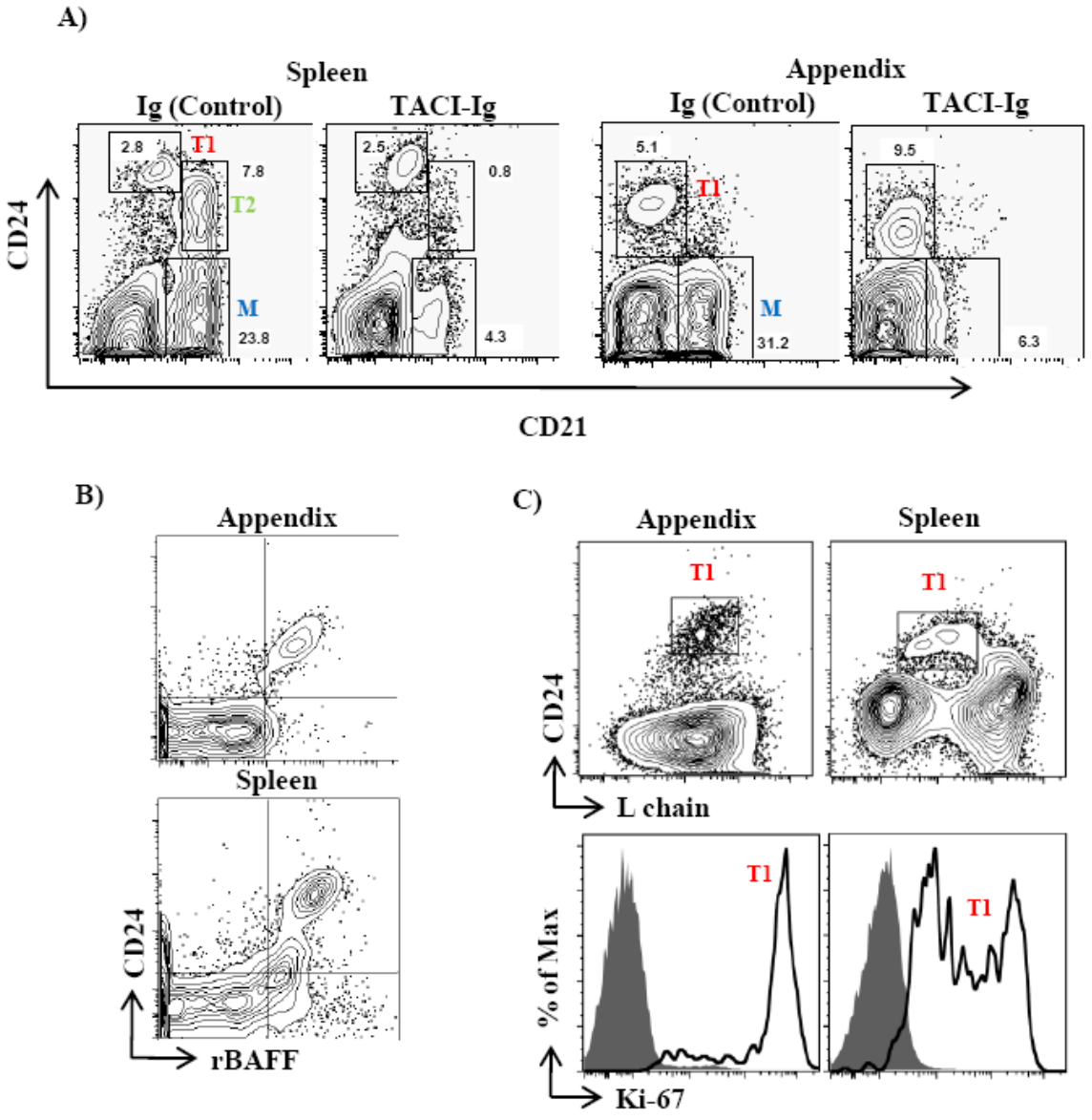
T1 = $CD24^{hi}CD21^{lo}$ T2 = $CD24^{hi}CD21^{+}$

ND = Not detectable

together, these results demonstrate that in adult rabbits, immature B cells can be phenotypically delineated into two transitional B cell subsets, similar to murine T1 and T2 B cells. The frequencies of these cells in different tissues are shown in Table 3

Functional analysis of transitional B cells

In vivo, murine transitional B cells require BAFF for maturation into B cells, and in the absence of BAFF, peripheral B cell development is blocked at the T1 stage (Schiemann et al., 2001). If the $CD24^{hi}CD21^{lo}$ B cells in rabbit are in fact T1 cells and require BAFF for maturation, then I predicted that the absence of BAFF would block peripheral B cell development at the T1 stage. I therefore neutralized BAFF by injecting newborn rabbits with a soluble decoy receptor (TACI-Ig) and found, as predicted, a dramatic decrease in splenic T2 and mature B cells, while the T1 B cell population remained intact (Fig 17A *left*). Similarly, in the appendix, the mature B cell population was eliminated by neutralization of BAFF, but the $CD24^{hi}$ transitional B cell population was not reduced, but instead appeared to accumulate (5.1% vs 9.5%)(Fig 17A *right*). These data indicate that $CD24^{hi}CD21^{lo}$ B cells are murine-like T1 cells that require BAFF for their maturation. Further, the absence of splenic T2 and mature B cell compartments upon TACI-Ig treatment suggests to me that T1 cells give rise to T2 cells, which in turn likely develop into mature B cells. These data also indicate that GALT T1 cells are functionally similar to splenic T1 cells in their requirement for BAFF to differentiate into mature B cells that occupy the B cell follicles.


A)
Spleen
Ig (Control)
TACI-Ig
Appendix
Ig (Control)
TACI-Ig
CD24
CD21
T1
T2
M
2.8
7.8
23.8
2.5
0.8
4.3
5.1
31.2
9.5
6.3
B)
Appendix
Spleen
CD24
rBAFF
C)
Appendix
Spleen
T1
CD24
L chain
% of Max
Ki-67

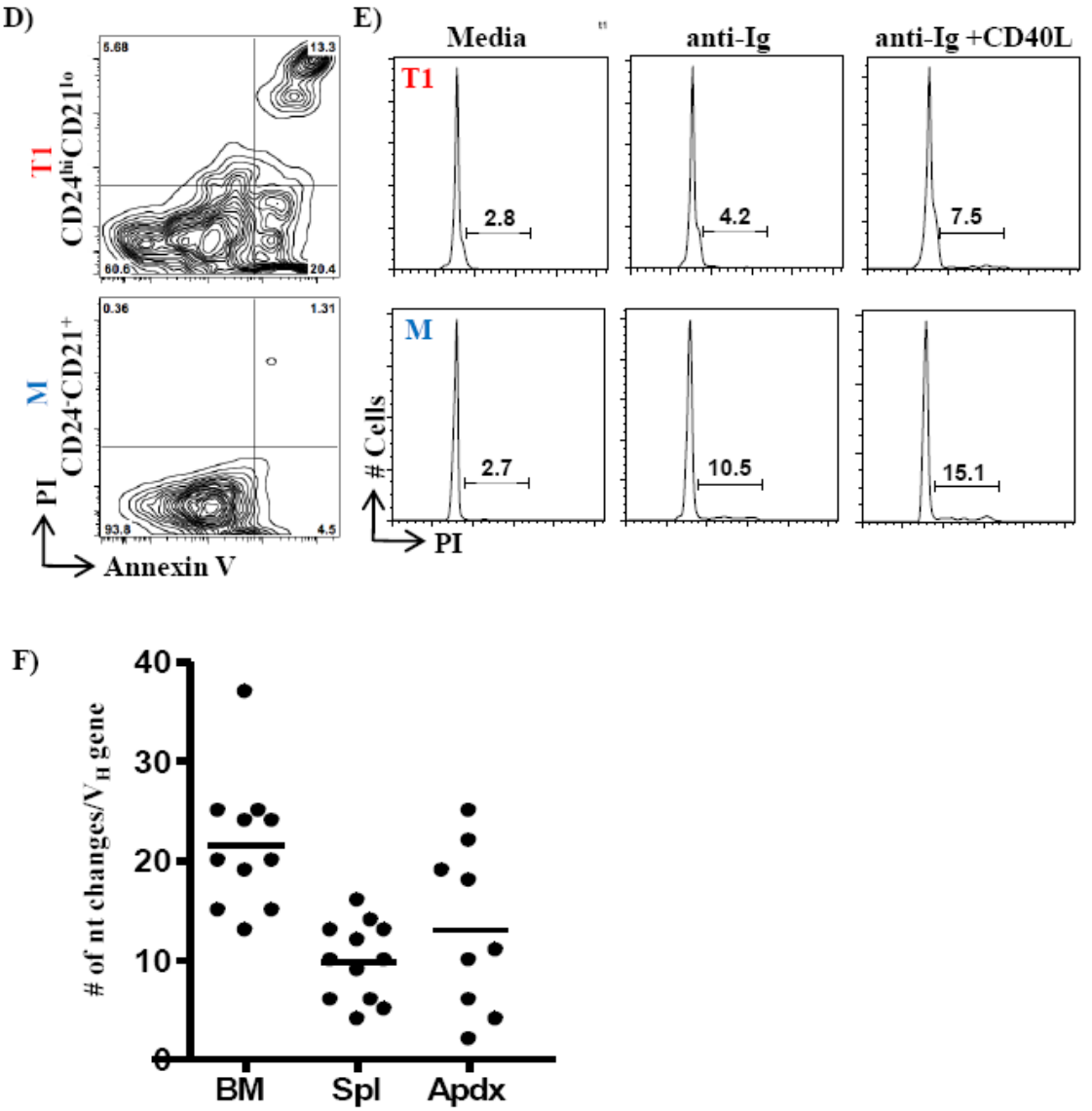


Figure 17: Functional analysis of transitional B cells. Flow cytometric analysis of appendix and spleen cells stained with A) anti-CD21 and anti-CD24 in TACI-Ig-treated and control (Ig) rabbits, B) Anti-CD24 and rBAFF, C) *upper*: anti-CD24 and anti-L chain; *lower*: anti-Ki67 (open histograms) of T1 cells from upper diagram. Shaded histogram = isotype control. Flow cytometric analysis of FAC-sorted splenic T1 (*upper*)

and mature B cells (*lower*) stained with D) Annexin V and propidium iodide (PI) after 12-15 hrs in culture with anti-Ig, or E) PI to determine the frequency of cycling cells after 24 hrs in culture either alone (media), with anti-Ig or anti-Ig plus CD40L (see materials and methods). The numbers in the plot indicate the percentage of cells in the S and G2 phase. F) Somatic diversification of V regions of PCR-amplified VDJ genes from splenic and appendix T1 B cells. The horizontal bar represents average number of nucleotide (nt) changes/V_H gene (excluding D and J regions); each dot represents one V_H gene sequence. Sequences obtained from two adult rabbits are shown. Data in B) to E) are representative of 2-3 independent experiments. Data in A) are representative of two control and three TACI-Ig treated rabbits.

As shown in Fig 11A, most freshly isolated B cells in rabbit do not bind to rBAFF. But, do transitional B cells bind BAFF? Because the $CD24^{hi}$ T1 B cells were also IgM^{lo}, I tested if they represented the $rBAFF^{+}IgM^{lo}$ subset that I identified in neonatal rabbits (Fig 15A). I found that most BAFF-binding cells in the appendix and spleen were $CD24^{+}$ B cells, indicating that transitional B cells bind BAFF (Fig 17B). Further, consistent with the localization of rBAFF-binding cells in the proliferation ($Ki\text{-}67^{+}$) region of B cell follicles in the appendix, I found T1 B cells in both appendix and spleen were $Ki\text{-}67^{+}$, indicating that they are cycling *in vivo* (Fig 17C). To functionally characterize transitional B cells, I tested how they responded to anti-Ig stimulation *in vitro*. Following anti-Ig treatment of FAC-sorted splenic T1 and mature B cells, T1 cells did not progress into cell cycle, but instead underwent apoptosis (Fig 17D *top left*), while the mature B cells readily entered cell cycle (Fig 17E *middle*). However, upon co-stimulation with CD40L, T1 cells did enter cell cycle, but at a reduced level compared to mature B cells (Fig 17E, *right*). I also tested if the Ig genes in T1 B cells were somatically diversified. Murine transitional B cells are constantly replenished from the BM and consequently have unmutated Ig genes even in adults (Allman et al., 1993). Because new B cells are not made in BM of adult rabbits (Jasper et al., 2003, Crane et al., 1996), I predicted that T1 B cells in these rabbits would be diversified. I isolated T1 B cells from spleen and appendix of adult rabbits and PCR-amplified and sequenced the Ig VDJ genes. As expected, I found the IgH genes had undergone somatic diversification (Fig 17F), suggesting that the B cells had been through a GC-like reaction and were not recent emigrants from the BM.

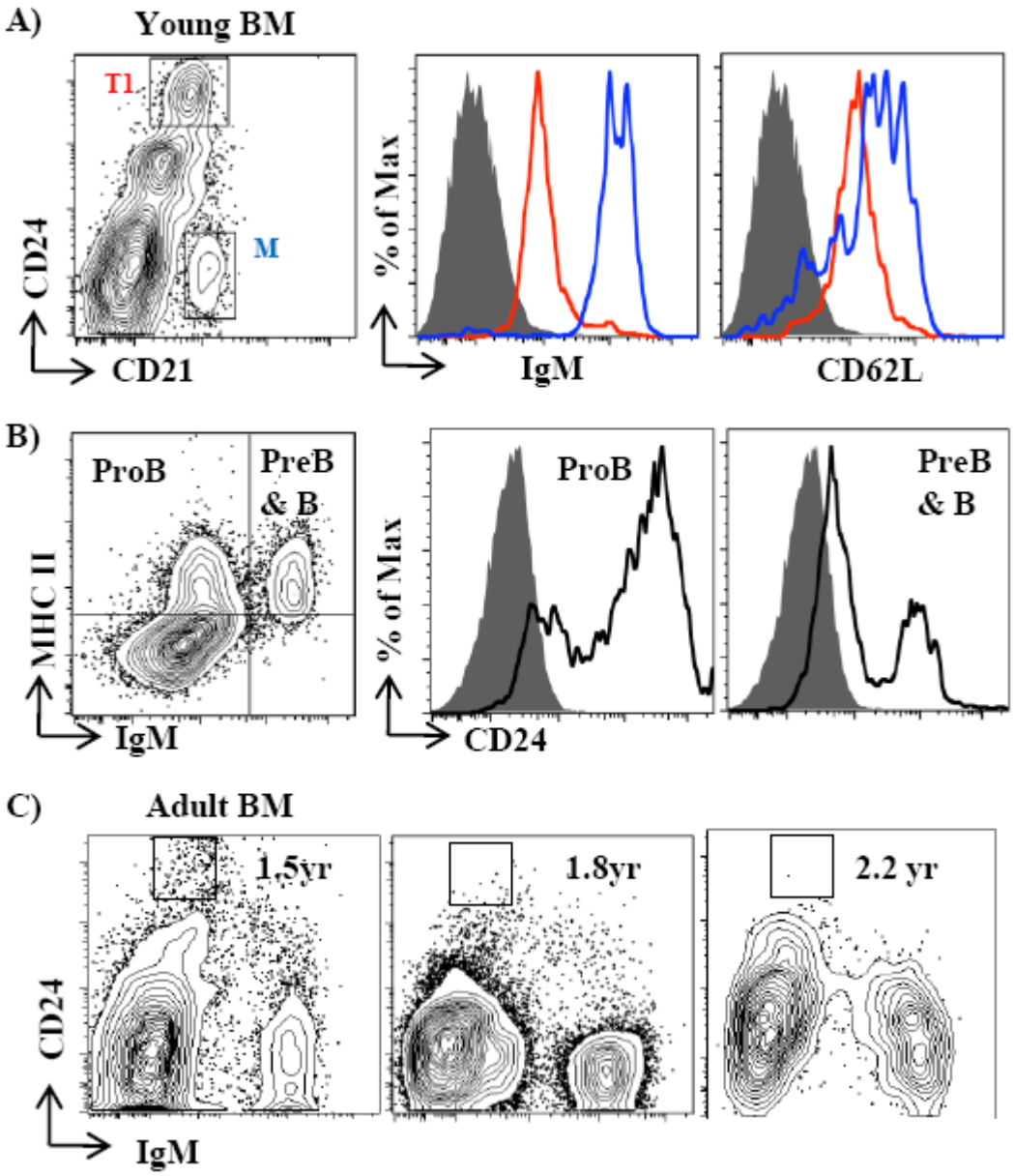


Figure 18: Flow cytometric analysis of T1 B cells in BM. A) BM cells from a young rabbit (8 weeks of age) stained for CD21 and CD24, with histograms of IgM and CD62L staining of cells in T1 (red) and M (blue) cell gates. The gray-filled histograms represent staining with an isotype control mAb. B) BM cells from a young rabbit stained for IgM,

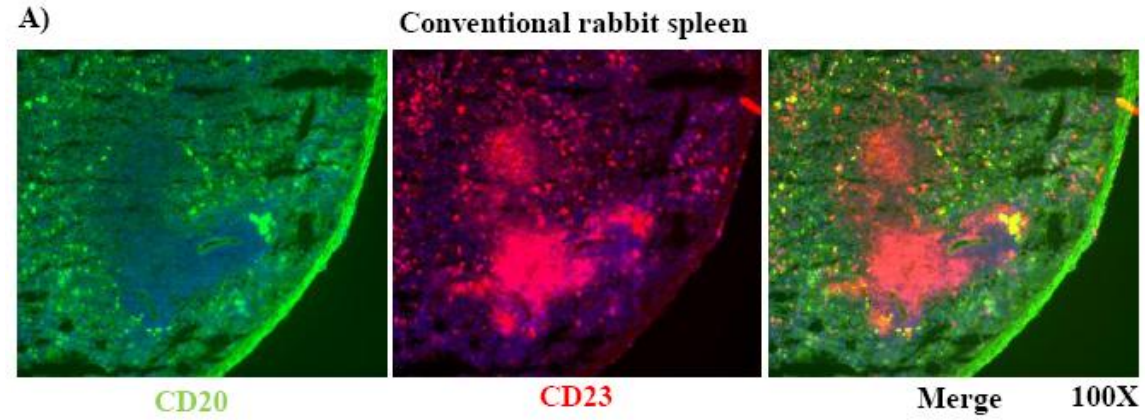
MHC II and CD24, with CD24 histograms of cells in proB and preB & B cell gates. The gray-filled histograms represent staining with an isotype control mAb. C) BM cells from adult rabbits stained for CD24 and IgM. All plots are representative of staining obtained from at least 3 rabbits.

Transitional B cells in the BM

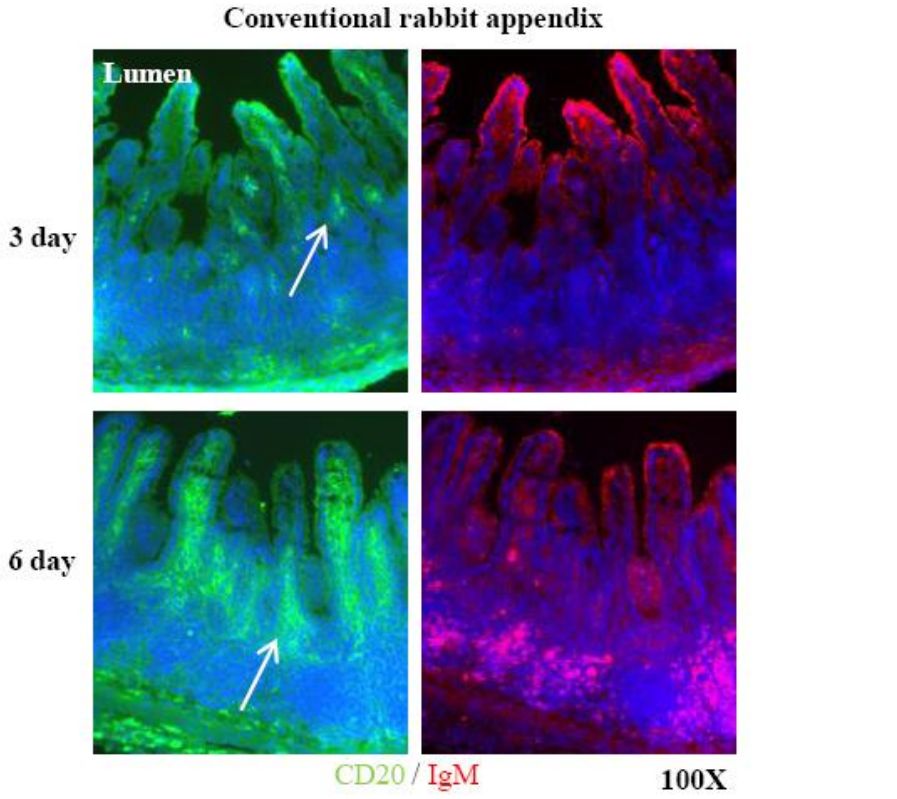
In young rabbits, where there is ongoing lymphopoiesis in the BM, I identified T1 B cells ($CD24^{hi}CD21^{lo}IgM^{lo}CD62L^{lo}$) (Fig 18A) and a $CD24^{lo}CD21^{-}$ population that I thought might include proB and preB cells. To test if proB and preB cells are $CD24^{+}$, I stained BM cells for MHC II and cytoplasmic IgM and found that proB cells and also cells in the preB & B cell gate (presumably preB cells) were $CD24^{+}$ (Fig 18B). In addition to early B cell progenitors, the $CD24^{lo}CD21^{-}$ population might include other lineages such as $CD24^{+}$ stromal cells and common lymphoid progenitors (Israel et al., 2005). In contrast to young rabbits, as expected, I found few, if any, $CD24^{hi}$ T1 B cells in the BM of adult rabbits (Fig 18C). Nucleotide sequence analysis of the VDJ genes of these T1 cells showed that the IgH genes were somatically diversified (Fig 17F), confirming that they were not newly-made B cells. I suggest that the few diversified T1 B cells in the adult BM may represent recirculating transitional B cells.

Tissue localization of transitional B cells

To determine the location of transitional B cells in spleen, I analyzed tissue sections by immunohistology. Because anti-CD24 did not stain frozen tissue sections effectively, I used anti-CD20, which binds all $CD24^{+}$ B cells (T1 and T2 B cells) (Fig 16A *lower*). By using anti-CD23 to label the follicular zone, I found that $CD20^{+}$ transitional B cells in spleen were located near the margins of the follicles and also in the red pulp (Fig 19A). These data are similar to the localization of splenic transitional B cells in mouse (Loder et al., 1999).


A)
Conventional rabbit spleen
CD20
CD23
Merge
100X
B)
IgH transgenic rabbit
PB
Spleen
Appendix
IgM
CD21
Lumen
T
T
CD20 / IgM
100X

C)


Conventional rabbit appendix
Lumen
3 day
6 day
CD20 / IgM
100X
Appendix dome
Lumen
CD20
IgM
Merge
200X

D)

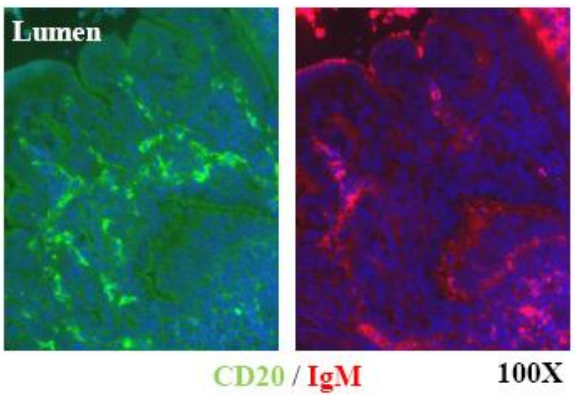

Lumen
CD20 / IgM
100X

Figure 19: Tissue localization of CD20$^+$ transitional B cells. A) Immunohistological staining of spleen (6-week old) section for CD20 (transitional B cells) and CD23 (follicular B cells); B) Flow cytometric and immunohistological analyses of tissues from an IgH Tg rabbit stained for IgM and CD21 (*top*), and CD20 and IgM (*bottom*), respectively. T = T cell areas. C) Staining for CD20 and IgM in appendix from normal 3 day and 6 day old rabbits (*top*). Arrows indicate CD20$^+$ cells in the dome and villi. Lower panel shows staining of a representative dome of the appendix from the 6 day old rabbit (200X). D) Staining for CD20 and IgM in the villous region of appendix from a normal 4 week old rabbit. Magnification = 100X or 200X

To localize transitional B cells in GALT during the early stages of B cell development, I examined the appendix from an IgH transgenic (Tg) rabbit. These IgH Tg rabbits are deficient in B cells early in ontogeny, but IgM^+ B cells accumulate gradually in the periphery during a span of several weeks to months. They appear first in the appendix and SR and later in PP, MLN, blood and spleen (Jasper et al., 2007). The delayed temporal appearance of B cells in these rabbits offered an opportunity to study the early stages of peripheral B cell development. I examined one IgH Tg rabbit that lacked IgM^+ B cells in the periphery (PB and spleen), but had a few B cells in GALT (Fig 19B *top*) and found that the IgM^+ B cells in the appendix were $CD21^{lo}$ (Fig 19B *top right*), and $CD20^+$ (Fig 19B *bottom*), suggestive of a transitional B cell phenotype. The B cells were scattered, predominantly in the domes and villi of underdeveloped B cell follicles located between large T cell areas (Fig 19B). IgM staining in the domes and villi was not detectable (Fig 19B), presumably due to low expression of IgM on $CD20^+$ transitional B cells. Similar to IgH Tg rabbits, I identified transitional B cells in the dome and villous regions of appendix from conventional neonatal rabbits (Fig 19C). At day 3 after birth, I found no identifiable IgM^+ B cells in the follicles, but found a few $CD20^+$ transitional B cells in the domes and villi. By day 6, these $CD20^+$ cells accumulated in number, along with IgM^+ follicular B cells (Fig 19C). Similar to neonates, I found $CD20^+$ cells located in the villous regions of the appendix of a 4 week old rabbit (Fig 19D). I conclude that during development, transitional B cells migrate to the domes and villi of the appendix prior to differentiating into follicular B cells. The unique and close proximity of GALT transitional B cells to the intestinal lumen suggests to me that these B

cells may interact with commensal bacteria or bacterial-derived products that promote B cell activation and maturation in GALT.

CHARACTERIZATION OF MATURE B CELL SUBSETS

The presence of transitional B cells in the domes and villi of both IgH Tg, and conventional neonatal rabbits, followed by the appearance of IgM^+ follicular B cells suggested to me that transitional B cells differentiate into follicular/mature B cells. I phenotypically and functionally characterized the mature B cell subsets from different lymphoid tissues.

Identification of follicular and MZ B cells in the spleen

Similar to identification of transitional B cell subsets in the previous section, I identified follicular and MZ B cells based on their localization and expression of several markers that are used to delineate mature B cell subsets in mice and humans. Because CD23 is a marker for mature B cells and expressed in the follicular zone, but not in the MZ of a B cell follicle (Wells et al., 1995), I used anti-CD23 mAb and identified two populations of B cells in the spleen: $CD23^-$ and $CD23^+$ (Fig 20A *top*) and found that the $CD23^+$ B cells were localized in the middle of a B cell follicle (Fig 20B *top*). Further, the $CD23^-$ B cells had higher levels of surface IgM compared to $CD23^+$ B cells (Fig 20A *top*). These data indicate that anti-CD23 mAb can be used as a marker to identify follicular zone B cells in rabbit. Further, the absence of CD23 staining around the follicles and the higher expression of IgM on $CD23^-$ B cells suggests that MZ B cells are

IgM^{hi} $CD23^{-}$, similar to the phenotype of rodent MZ B cells (Wells et al., 1995). Although MZ B cells are defined primarily on the basis of their anatomical localization, they are also identified based on the expression of a number of distinct markers. In mice, they are broadly defined as $CD23^{-}$, $CD1d^{+}$ (or $CD1c^{+}$ in humans) and $CD9^{+}$ (Pillai et al., 2005). Because mAbs to rabbit CD1b isoform and CD9 are available, and these reagents stain a subset of splenic B cells (Fig 20A *middle*), I tested whether CD1b and CD9 is expressed on B cells in the MZ. I stained spleen sections with either anti-IgM or L Chain Ab to identify B cell follicles (data not shown) and with anti-CD1b and anti-CD9 mAbs, and found both CD1b and CD9 expressed in the margins of B cell follicles (Fig 20B *middle*). CD9 expression was highest in the MZ, and was also expressed at lower levels in some T cell areas (Fig 20B and data not shown). Taken together, these data indicate that the MZ contains B cells that are $CD23^{-}IgM^{hi}CD9^{hi}$ and $CD1b^{+}$.

In humans, because IgM^{+} $IgD^{+}CD27^{+}$ B cells reside in the MZ, CD27 serves as a pan-marker for MZ B cells (Weill et al., 2009). Using an anti-CD27 mAb, I identified $CD27^{+}$ and $CD27^{-}$ B cells in the spleen and found that the $CD27^{+}$ B cells expressed higher levels of IgM compared to the $CD27^{-}$ B cells (Fig 20A *bottom*). By immunohistology, I found CD27 expressed along the margins of B cell follicles (Fig 20B *bottom*), indicating that CD27 also serves as a marker for rabbit MZ B cells. Further, I found that the $CD27^{+}$ gate included essentially all of $CD9^{hi}$ and $CD1b^{+}$ MZ B cells, and also contained $CD21^{hi}$ and $CD38^{hi}$ B cells, which are also characteristic of MZ B cells

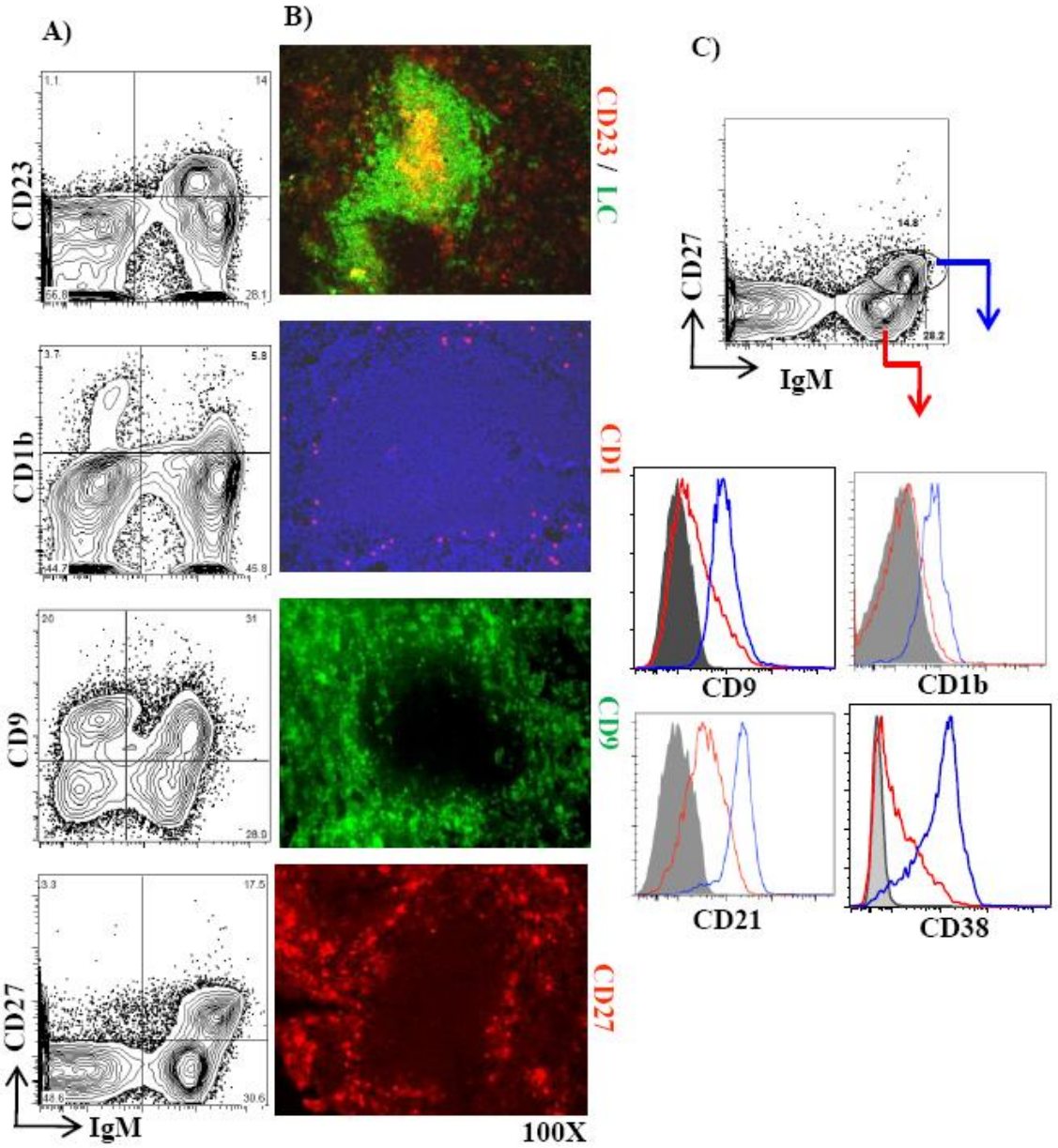


Figure 20: Identification of splenic MZ and follicular zone B cells A) Flow cytometric staining of spleen cells for CD23, CD1b, CD9 and CD27 B cells (IgM+). B) Immunofluorescent staining of spleen for CD23 and L-chain (LC) (*top*), CD1, CD9 (*middle*) and CD27 (*bottom*). C) Flow cytometric staining of splenic B cells for IgM and

CD27, with CD38, CD9, CD21 and CD1b histograms of cells in $IgM^{+}CD27^{+}$ gate (blue) and $IgM^{+}CD27^{-}$ gate (red). Shaded histograms = isotype controls. Magnification = 100X

(Fig 20C). Taken together, these data indicate, that similar to humans, CD27 also serves as a pan-marker to identify MZ B cells in rabbits.

Functional analysis of $CD27^+$ and $CD27^-$ B cells

Human marginal zone ($CD27^+$) B cells are large cells that secrete more Ig, and upon stimulation, enter cell cycle rapidly compared to $CD27^-$ B cells (Maurer et al., 1992, Agematsu et al., 1997). To examine if $CD27^+$ B cells in rabbit exhibit similar properties, I examined their size, and their response to stimulation with anti-Ig, CD40L and IL-4 *in vitro*. Both by flow cytometry and cytospin analysis, I found that $CD27^+$ B cells were larger in size compared to $CD27^-$ B cells (Fig 21A and B). *In vitro* stimulated FAC-sorted $CD27^+$ B cells exhibited approximately a 2-fold increase in total Ig secreted relative to stimulated $CD27^-$ B cells (Fig 21C). Further, following stimulation with anti-Ig, or co-stimulation with CD40L, I found a greater frequency of $CD27^+$ B cells in cell cycle compared to $CD27^-$ B cells (Fig 21D). Taken together, I conclude that $CD27^+$ and $CD27^-$ B cells represent functionally distinct mature B cell populations.

Mutational status of MZ B cells

One of the striking differences between rodent and human MZ B cells is that the Ig genes of rodent MZ B cells are unmutated, while human MZ B cells possess a diversified repertoire (Dammers et al., 2000, Dunn-Walters et al., 1995, Tierens et al., 1999). To test if MZ B cells in rabbits are somatically diversified, I FAC-sorted a subset of MZ B cells ($CD9^{hi}$) identified in figure 20 and analyzed the Ig V_H genes. Because I

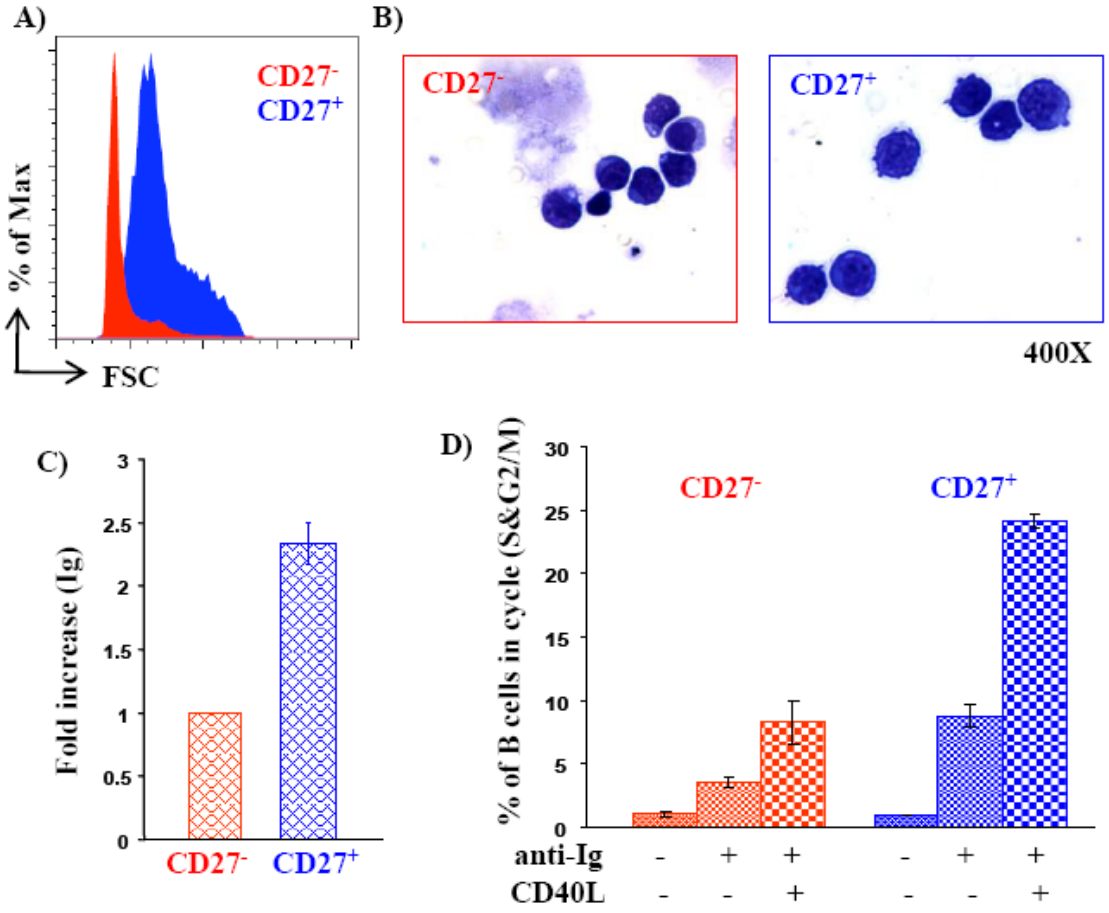
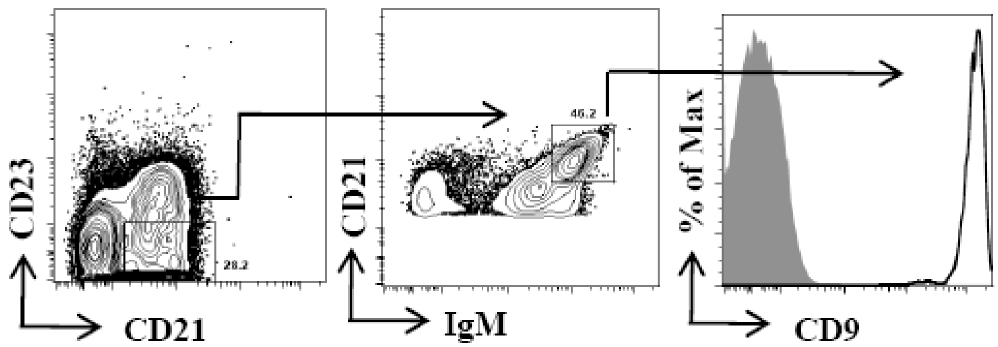


Figure 21: Functional analysis of splenic CD27$^+$ and CD27$^-$ B cells A) Histograms showing the forward light scatter (FSC) of CD27$^+$ (blue) and CD27$^-$ (red) B cells. B) H and E stained cytospins of FAC-sorted CD27$^+$ and CD27$^-$ B cells. Magnification = 400X. C) Bar graph showing the fold-increase in total Ig (as measured by ELISA) in the culture supernatants of FAC-sorted CD27$^+$ B cells, relative to CD27$^-$ B cells, which were both stimulated *in vitro* for 8 days with murine CD40L and human IL-4 (see materials and methods). The error bars = SEM calculated from fold changes obtained from three independent experiments. D) Bar graph showing the frequency of cycling CD27$^+$ and

$CD27^-$ B cells (as assessed by flow cytometry) stimulated for 24 hours with anti-Ig alone or co-stimulated with CD40L. The data are representative of two independent experiments and the error bars = SEM.

A)



B)

```
                                                                                   FR1
VH1a1                                                      gtgtccagtgtcagtcggtggaggagtccgggggtcg
15625                                                      .....................................
15624                                                      .....................................
15623                                                      .....................................
15622                                                      .................c...................
15620                                                      .....................................
15619                                                      .....................................
15618                                                      .................c...................
15617                                                      .....................................

                                                                                    CDR1
VH1a1 cctggtcacgcctgggacacccctgacactcacctgcacagtctctggattctccctcagtagctatgcaatgagctgggtccgc
15625 .........................................c....................c..a.ctac...............
15624 .................................................a..ga..................g...........
15623 .........................................c....................ga...ctac...............
15622 .........................................c..........a..a..........ccac...g...........
15620 .................................................a..ga........t.........t.c..........
15619 ...............................................................cg...c......a..........
15618 ...............agg.t.............................a..ga..........a....................
15617 .........................................c..........a..a..........ccac...g.....t.....

                  FR2                                               CDR2
VH1a1 caggctccagggaaggggctggaatggatcggaatcattag---tagtagtggtagcacatactacgcgagctgggcgaaa
15625 .................c.................g.....   ..c.g....................c.....t....
15624 .........................ac.............---..................c.................
15623 .................a...c...........ta.....aa      .a.a...at........................
15622 ............g....................t.....ta   ..c.g........g..gc....t...a......a...
15620 ........................a........g.....t.gac....g.---a.c....ac.................g.
15619 .......................................g.   ....g........gg.gc........c..........
15618 ............g.....................c......   ....g.......g........................
15617 .........................ac......ta.....a   ..c.g........g............c....t.a...

                                          FR3
VH1a1 ggccgattcaccatctccaaaacctcg---accacggtggatctgaaaatcaccagtccgacaaccgaggacacggccacctatttc
15625 ...........................   ....................g.....c.t...........................
15624 ...........................---.....................g..................................
15623 ...........................   .................g........c...g.........................
15622 ...................g.......   ....................g...................................
15620 .....................g.....   ..................t.g...................................
15619 ............g.......g.....atcg.....................g..................................
15618 ...........................   ...............gc.................t.....................
15617 ...........................   ....................g.......t...........................


VH1a1 tgtgccaga
15625 .........
15624 .........
15623 .........
15622 .........
15620 .........
15619 .........
15618 .........
15617 ........g
```

Figure 22: Sequence analysis of Ig genes from splenic marginal zone B cells A) Flow cytometric analysis of splenic $CD23^{-}$ $CD21^{hi}$ IgM^{hi} B cells for CD9 histogram (open). Shaded histogram = isotype control. B) Somatic diversification of V regions of PCR-amplified VDJ genes from FAC-sorted splenic $CD23^{-}CD21^{hi}IgM^{hi}CD9^{hi}$ B cells. Eight sequences compared to germline V_H1 gene sequence are shown.

performed this experiment before I identified the cross-reactive anti-CD27 mAb, I used anti-CD23, anti-IgM and anti-CD9 mAbs to define MZ B cells. I FAC-sorted $CD23^{-}$ $IgM^{hi}CD9^{hi}$ B cells (Fig 22A), which represents approximately 20% of the B cells in the spleen, and found that the Ig genes were somatically mutated (Fig 22B). From these data, I conclude that $CD9^{hi}$ MZ B cells in rabbit possess a diversified repertoire.

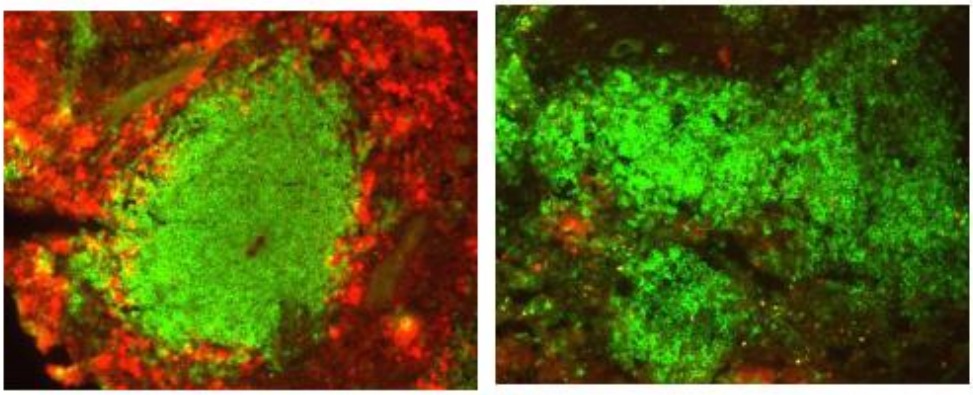
Analysis of the splenic B cell compartment in adult GALTless rabbits

Vajdy et al. (1998) found approximately a 50% decline in the percentage of B cells in rabbits in which all the organized GALT was surgically removed at birth. Are particular subsets of mature B cells affected by the removal of GALT? Because frozen tissues of the spleen from some of these GALTless rabbits were available, I analyzed them by immunohistology and found a lack of staining for CD27 in the MZ (Fig 23A), suggesting that MZ B cells were absent. I conclude that in the absence of GALT, the splenic $CD27^{+}$ MZ B cells do not develop. Using anti-CD23, I found the follicles in GALTless rabbits were all essentially $CD23^{+}$, while the B cell follicles in age-matched control rabbits had clearly distinct $CD23^{+}$ and $CD23^{-}$ areas (Fig 23B). Further, the follicular zone, as defined by $CD23^{+}$ staining appeared larger in size in GALTless rabbits when compared to controls (Fig 23B). This increase in the size of the follicular zone could be due to an expansion of the $CD23^{+}$ B cells in the absence of $CD27^{+}$ MZ B cells. Further, the absence of these MZ B cells likely explains the reduction in the frequency of B cells following removal of GALT at birth.

A)

Marginal zone

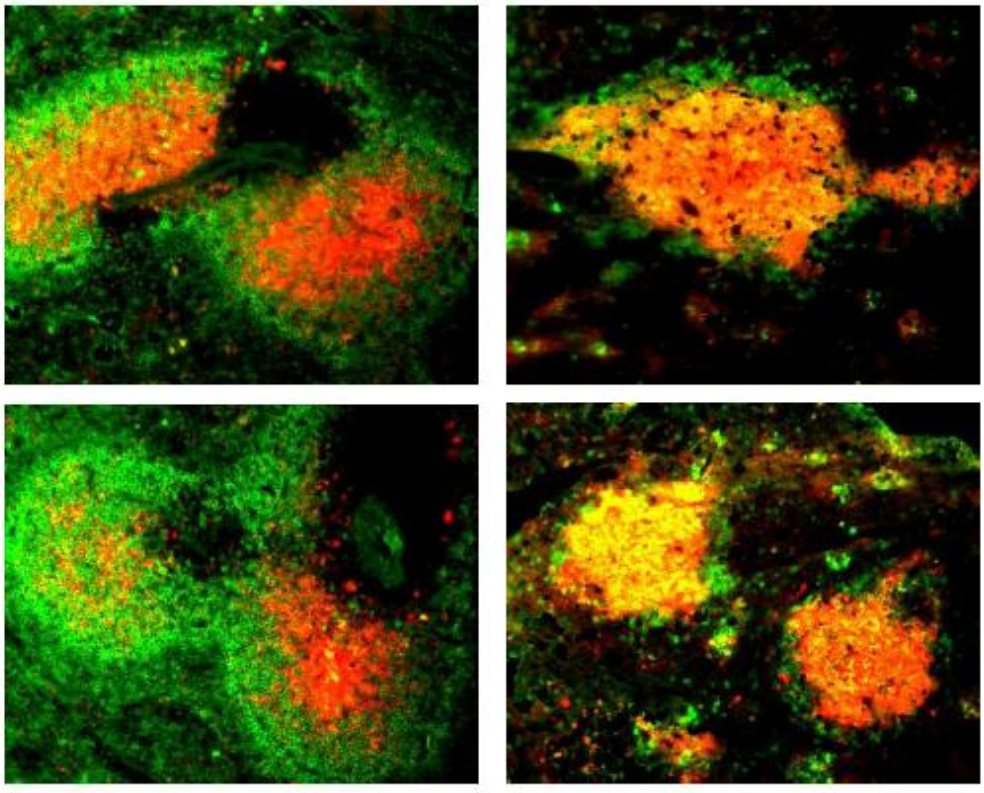
Control GALTless



LC CD27 100X

B) Follicular zone

Control GALTless



LC CD23 100X

Figure 23: Immunohistological analysis of spleen from GALTless and control rabbits. Staining of spleen section from litter-mate control and GALTless rabbit for A) L-chain (LC) and CD27 and B) LC and CD23. The two panels in B) represent different areas of the tissue. The data are representative of staining obtained from 3 GALTless and control rabbits.

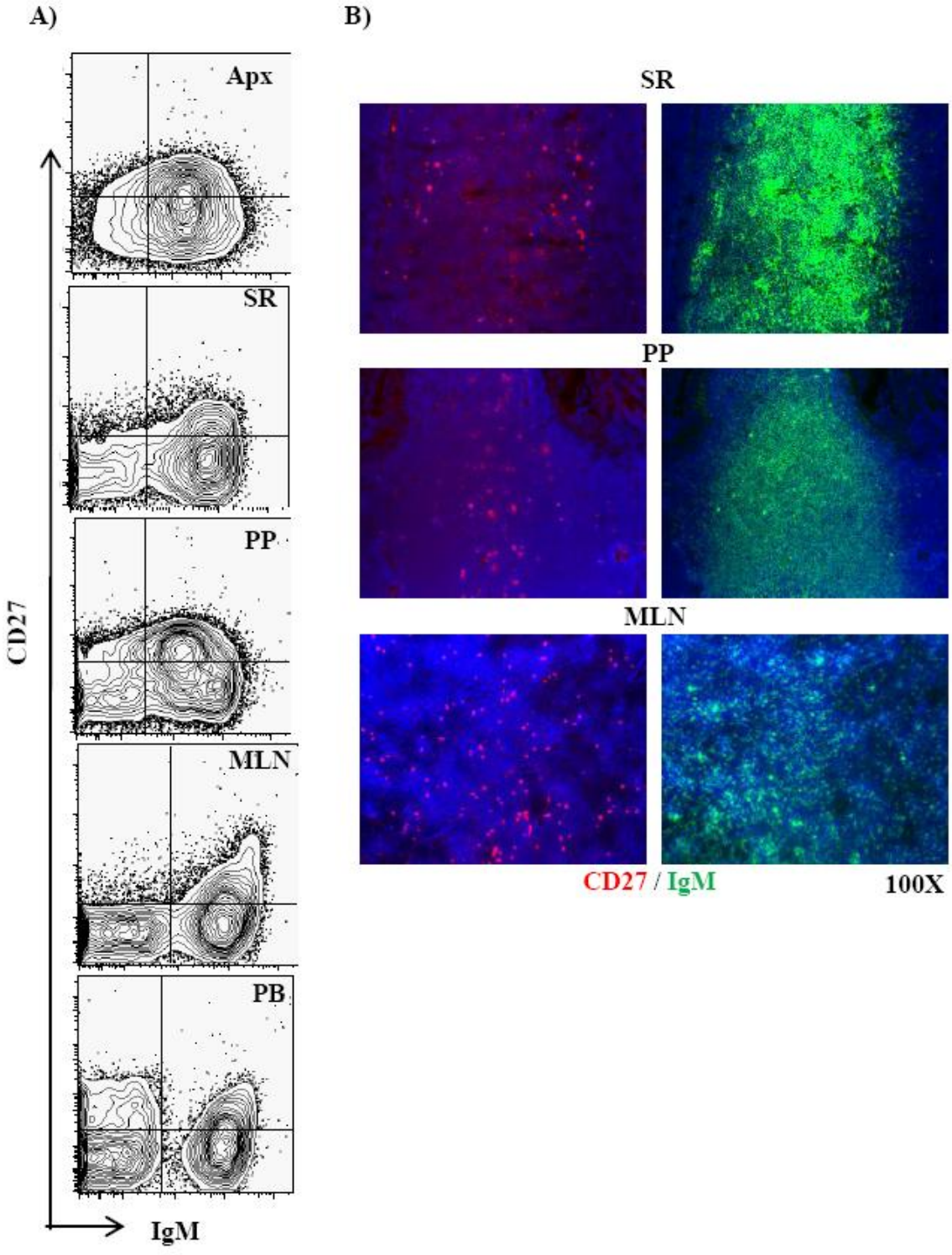

A)
Apx
SR
PP
MLN
PB
CD27
IgM
B)
SR
PP
MLN
CD27 / IgM
100X

Figure 24: Analysis of CD27^{+} B cells in GALT and peripheral blood. A) Flow cytometric staining of B cells from Apx, SR, PP, MLN, and PB with anti-IgM and CD27. B) Immunohistological staining of SR, PP, and MLN sections for IgM and CD27

MZ B cells in GALT and PB

MZ B cells in rodents are restricted to the spleen, while in humans, they are circulating and present in several lymphoid tissues, including GALT (Steiniger et al., 2005, Weill et al., 2009). Since MZ B cells do not develop in the absence of GALT, I asked if these B cells could be derived from, and therefore present in GALT and other lymphoid tissues. By flow cytometry, I found $CD27^+$ B cells in Apx, SR, PP, MLN and PB (Fig 24A). By immunohistology, I found these B cells scattered in the follicles of SR, PP and MLN (Fig 24B). I conclude that similar to humans, $CD27^+$ MZ B cells in rabbit are present in GALT and likely circulating.

Expression of Notch2 and its ligand Jagged-1 in GALT

Studies using gene knock-out models in mice revealed that signaling through notch2 and its ligands are required for MZ B cell development (Saito et al., 2003, Hozumi et al., 2004). However, the site(s) where these notch2-ligand(s) interactions occur is not clear (Pillai et al., 2005). Based on the absence of splenic MZ B cells in GALTless rabbits, I hypothesized that notch-ligand interactions may occur in GALT. Because introduction of commensals into GF appendices promote B cell development in GALT, these tissues can be used to study the early stages of B cell development. I examined if notch and its ligands are expressed in the reconstituted GF appendices, and in support of my hypothesis, I found notch2 transcripts in the B cell follicles of GF appendices into which *B. subtilis* and *B.fragilis* or *B. fragilis* alone were introduced (Fig 25A). I also found Jagged-1, one of the notch2 ligands expressed near the FAE (Fig 25B).

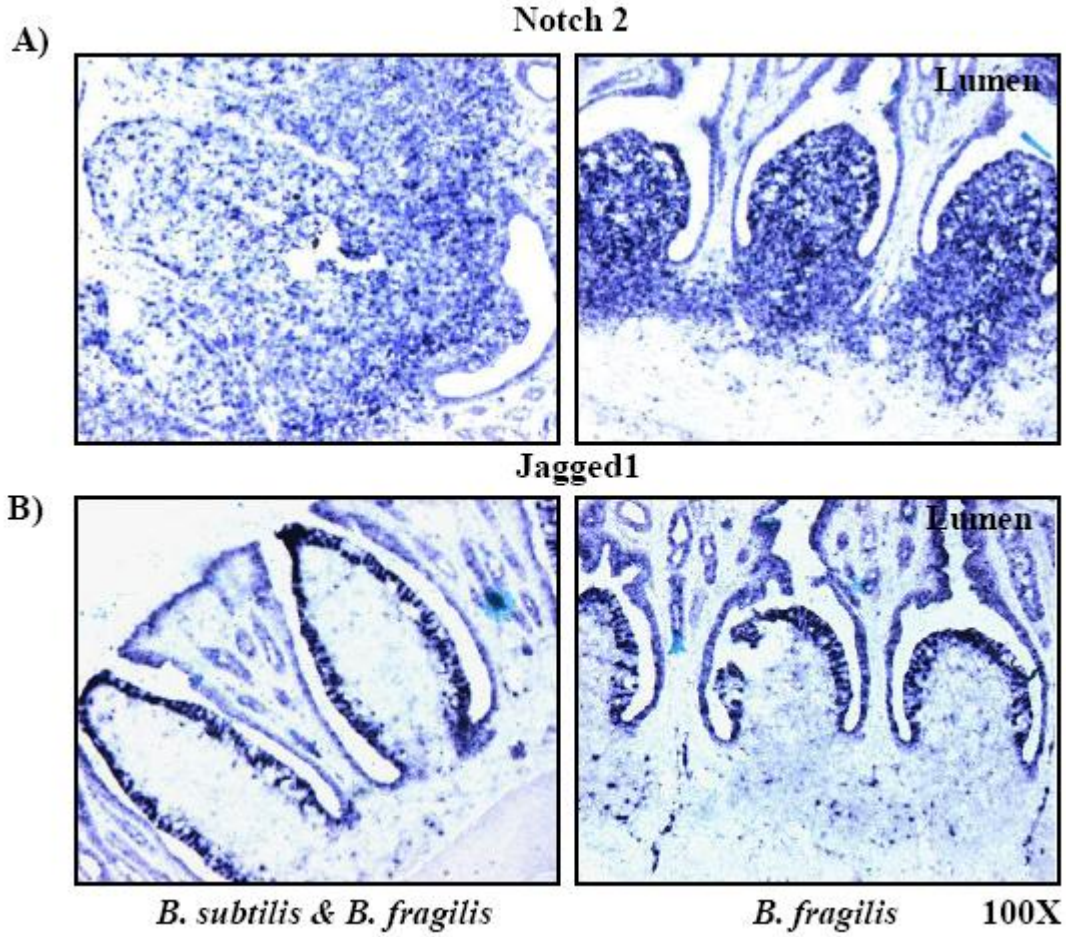
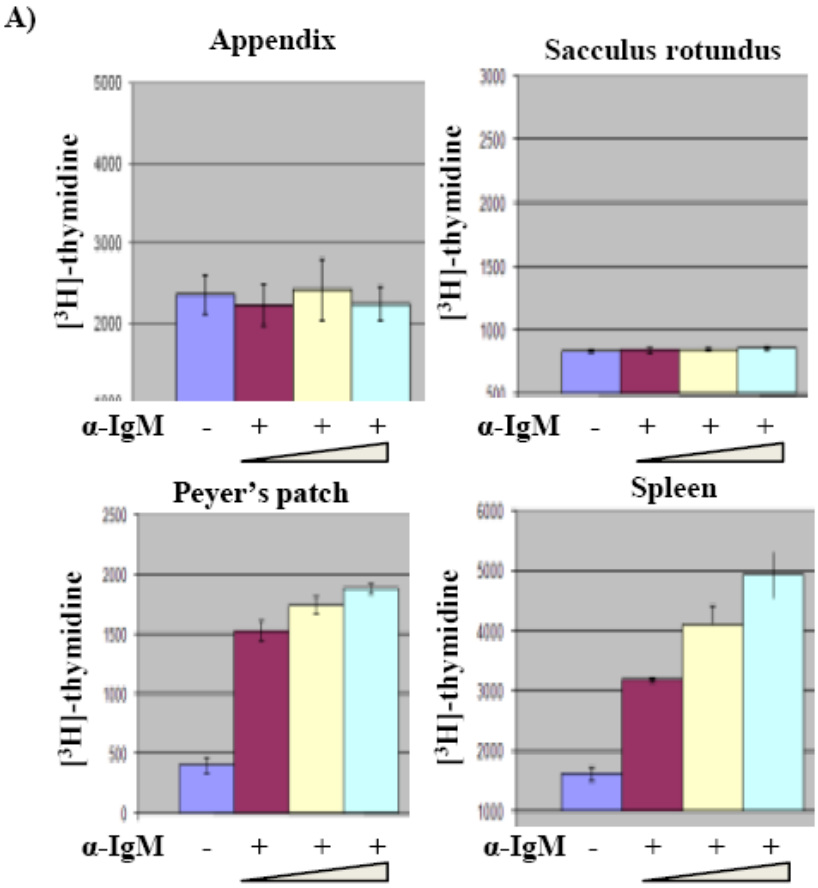


Figure 25: Expression of notch2 and jagged1 transcripts in appendix. *In situ* hybridization to detect notch2 (A), and Jagged1 (B) transcripts in the appendices from GF-appendix rabbits into which commensals; *B. subtilis* & *B. fragilis* or *B. fragilis* alone were introduced. The *in situ* hybridizations were performed by Dr. Dennis Lanning. Magnification = 100X.

This unique distribution of Jagged-1 near the FAE is reminiscent of the localization of transitional B cells in the domes and villi (Fig 19C), and in close proximity to the epithelial cells and commensal bacteria. As I discuss later, perhaps, transitional B cells that engage with notch ligands in these sites are induced to differentiate in to MZ B cells.

Phenotypic and functional analysis of B cells in GALT

The GALT in rabbits is comprised of several tissues: appendix, sacculus rotundus, Peyer's patch and mesenteric lymph nodes. However, B cells from the appendix are considered representative of GALT B cells and often, are the only GALT B cells studied. Consequently, it is not known if phenotypically and functionally distinct B cell subsets reside in the different compartments of GALT. To understand the nature of B cells in these different tissues, I first examined their response to anti-Ig stimulation, with the expectation that mature follicular B cells in these tissues will proliferate upon BCR crosslinking, while immature or GC B cells will not proliferate, but undergo apoptosis. Following stimulation with anti-Ig, I found that PP B cells proliferated in a dose-dependent manner, whereas appendix and SR B cells did not (Fig 26A). As a positive control for proliferation, I stimulated splenocytes with anti-Ig, and as expected found a dose-dependent proliferation (Fig 26A). Further, 24 hours after BCR crosslinking, I found that a large fraction of appendix B cells were caspase3^{+} (Fig 26B *top*), indicating that those B cells were undergoing apoptosis. In contrast, only a small subset of splenic B cells, (IgM^{lo}) presumably transitional B cells were caspase3^{+} (Fig 26B *bottom*). The lack of a proliferative response of appendix B cells to anti-Ig stimulation, and the induction of


A)
Appendix
Sacculus rotundus
Peyer's patch
Spleen
[3H]-thymidine
α-IgM - + + +

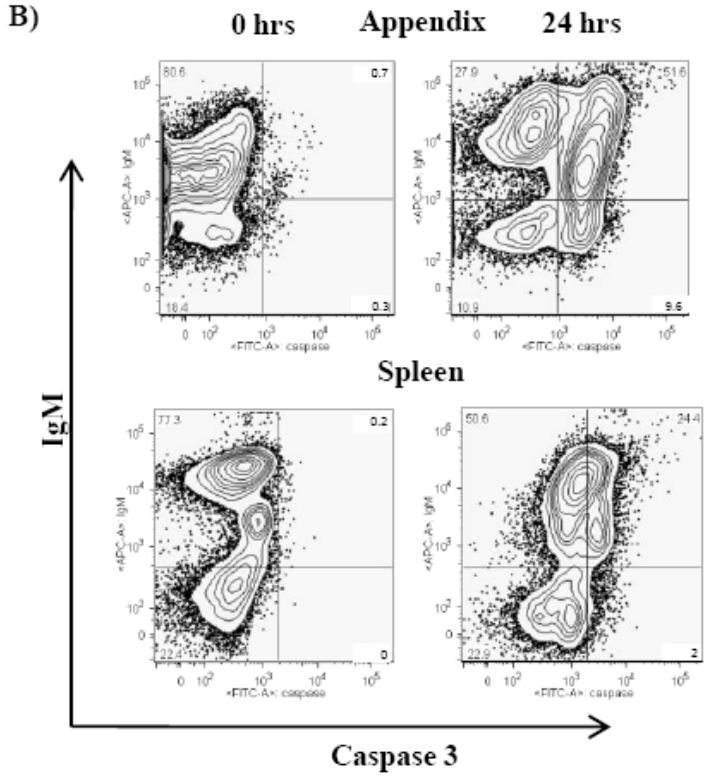
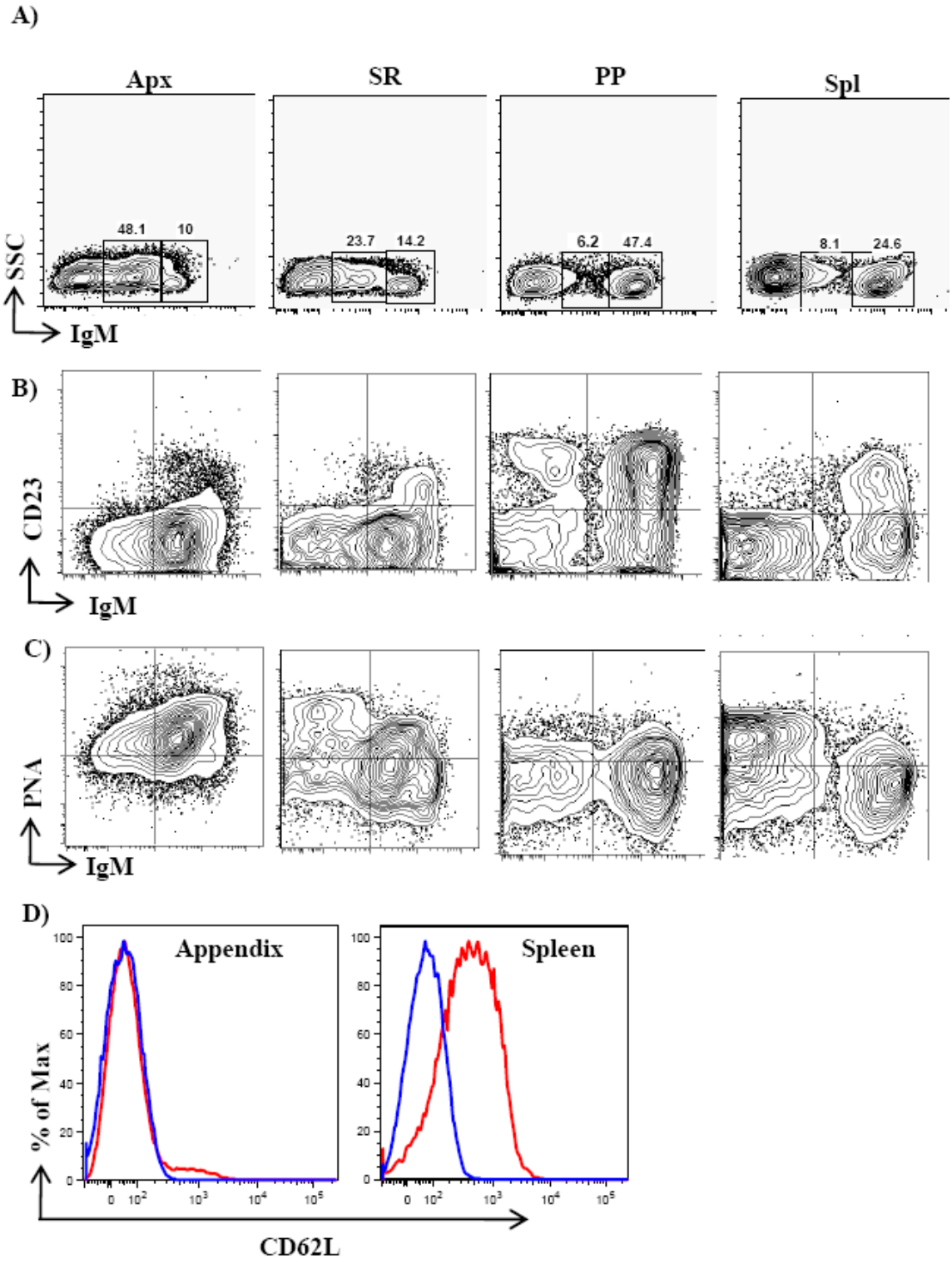

B)
0 hrs
Appendix
24 hrs
Spleen
IgM
Caspase 3

Figure 26: Functional analysis of B cells from various compartments of GALT. A) Proliferation of B cells isolated from appendix, sacculus rotundus, Peyer's patches and spleen, cultured for 3 days in the presence of anti-Ig. Cells were pulsed with [^{3}H]-thymidine during the last 18 hrs in culture. The open triangles indicate increasing concentrations of anti-Ig (5, 10, 20 μg/ml). B) Staining of B cells from appendix and spleen for IgM and caspase 3, 0 and 24hrs after BCR crosslinking.

apoptosis under those conditions, suggested to me that the appendix was composed of GC B cells, which are known to respond poorly to BCR crosslinking (Holder et al., 1991). To further characterize these GC-like cells, I analyzed B cells from various compartments of GALT using markers that are characteristic of GC B cells in other species. Based on the expression of surface IgM, I broadly classified GALT B cells as IgM^{hi} or IgM^{lo}. In the appendix and SR, the IgM^{lo} B cells predominated, whereas most B cells in the PP were IgM^{hi} (similar to B cells in the spleen) (Fig 27A). Because GC B cells downregulate CD23 and express high levels of PNA (Butch and Nahm, 1992, Ingvarsson et al., 1999), I stained cells for these markers and found that most B cells in the appendix were $CD23^{lo}PNA^{hi}$ (Fig 27B and C), indicative of a GC B cell phenotype. The SR B cells also appeared to be primarily GC B cells because they were mostly $CD23^{-}PNA^{lo/int}$ (Fig 27B and C) and also because they did not proliferate in response to anti-Ig treatment (Fig 26A). In contrast to appendix and SR, the PP B cells appear to be primarily $CD23^{+}PNA^{lo}$ mature B cells, similar to the B cells found in spleen (Fig 27B and C). Another characteristic feature of GC B cells is that they are largely non-recirculating, and therefore $CD62L^{-}$, and express BCL6 (Reichert et al., 1983, Cattoretti et al., 1995). Accordingly, I found essentially all appendix B cells were $CD62L^{-}$, while mature B cells in the spleen were largely $CD62L^{+}$ (Fig 27D). Further, I found BCL6 expressed predominantly in the dark zone-like areas (Fig 27E *top*) of the appendix where $CD79a^{+}$ $LC^{-/lo}$ B lineage centroblasts like cells were located (Fig 27E *bottom*).


A)
Apx
SR
PP
Spl
48.1
10
23.7
14.2
6.2
47.4
8.1
24.6
SSC
IgM
B)
CD23
IgM
C)
PNA
IgM
D)
Appendix
Spleen
% of Max
CD62L

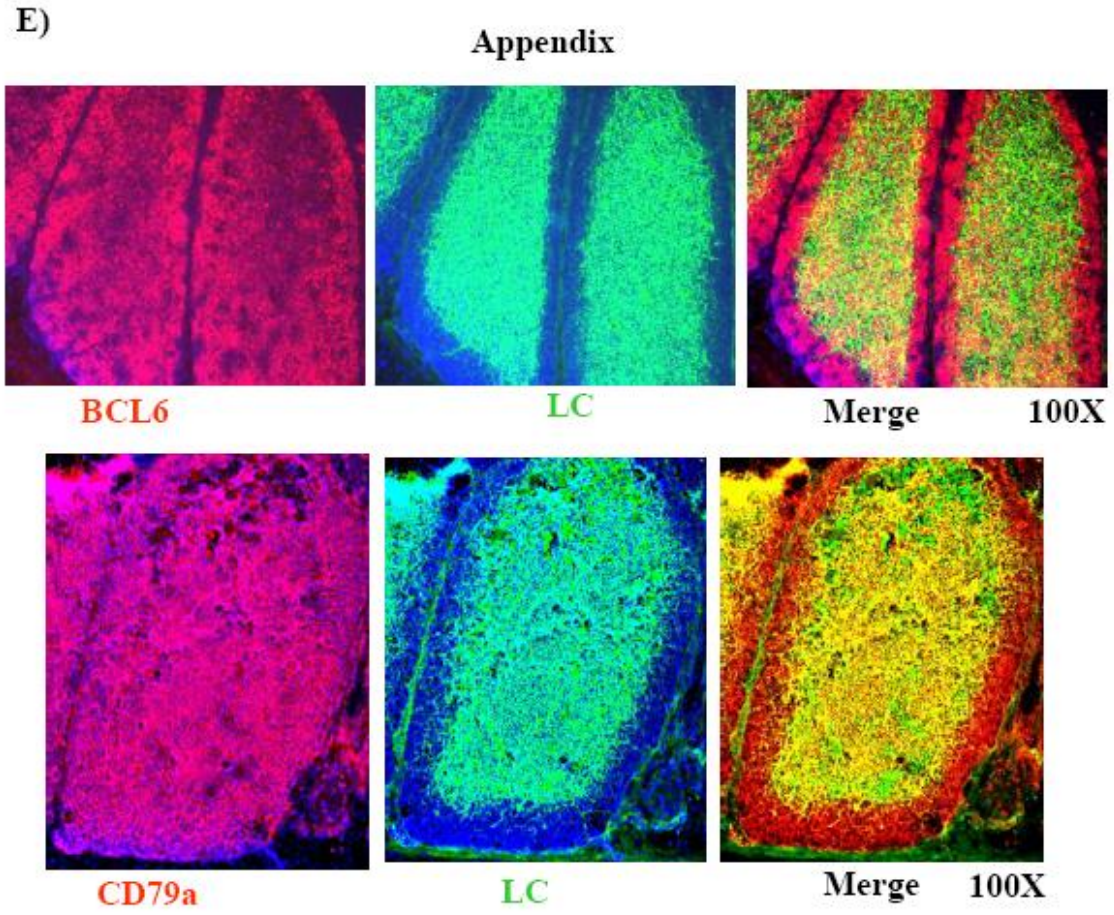


Figure 27: Phenotypic analysis of B cells from various compartments of GALT.

Flow cytometric analysis of B cells from Apx, SR, PP, and spleen for IgM (A), CD23 and IgM (B), PNA and IgM (C). D) Histogram (red) showing the expression of CD62L on IgM^+ (gated) B cells from the appendix and spleen. Blue histograms = isotype control. E) Immunohistological staining of appendix section for BCL6 and LC (*top*) and CD79a and LC (*bottom*). Magnification = 100X.

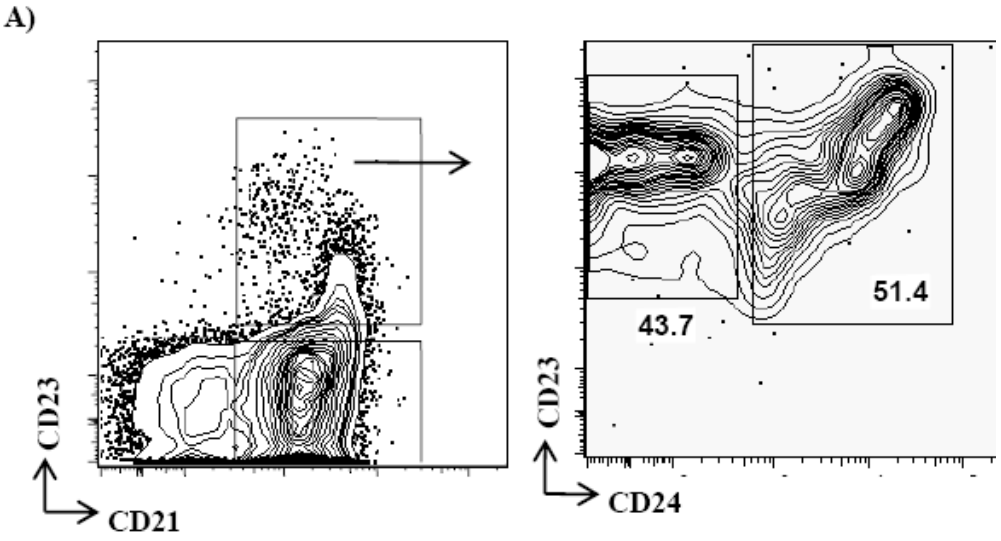
Taken together, I conclude that most B cells in the appendix and SR are GC B cells that are largely non-recirculating. Further, because PP B cells exhibited characteristics similar to splenic B cells, I conclude that PPs are comprised of mature follicular B cells. These findings reveal a previously unappreciated difference in the composition of B cells residing in distinct anatomical locations of the gut; large intestine (Appendix and SR) and small intestine (PP).

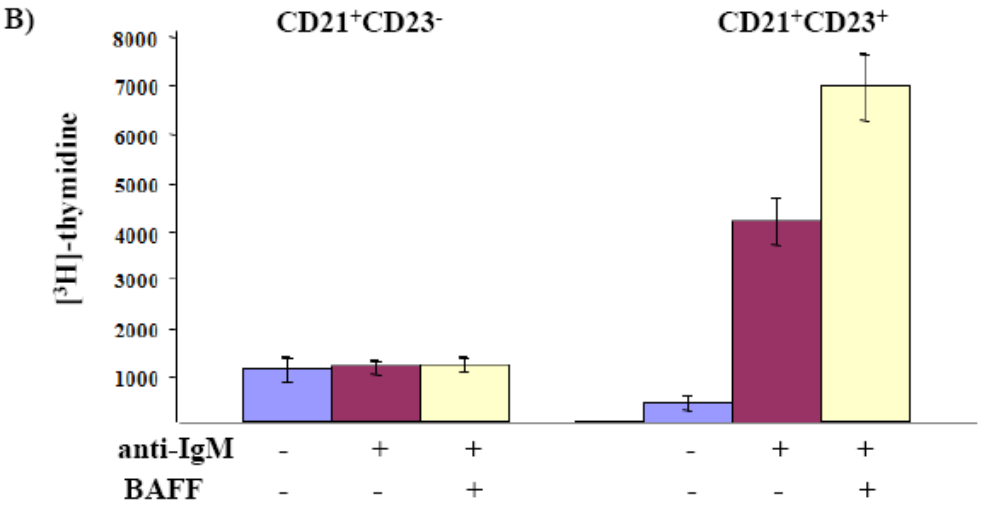
Identification of a small subset of mature B cells in the appendix

Although most B cells in the appendix were $CD23^-$ GC B cells, I observed a small subset of B cells (less than 5%) that were $CD23^+$ (Fig 27B and 28A *left*). Further, I found that approximately, 50% of these $CD23^+$ cells were $CD24^+$ (Fig 28A *right*), indicating that cells within the $CD23^+$ population include both $CD24^-CD23^+$ mature B cells and $CD24^+CD23^+$ transitional B cells. To determine if the $CD24^-CD23^+$ B cells were indeed mature B cells, I FAC-sorted $CD23^+$ B cells and found that upon stimulation with anti-Ig and BAFF, they proliferated, while the $CD23^-$ cells did not (Fig 28B), but instead underwent apoptosis (Fig 28C). These results indicate that the small numbers of $CD23^+$ B cells in the appendix and presumably, in the SR are mature B cells.

Expression of activation markers on appendix and splenic B cells from adult rabbits

As reviewed in chapter 1, peripheral B cells in mice are constantly replenished by the BM. When B cell production in the BM of mice was experimentally ablated, no conventional B2 B cells were found. However, a small number of B cells (MZ and B1)


A)
CD23
CD21
CD23
CD24
43.7
51.4


B)
CD21+CD23-
CD21+CD23+
[3H]-thymidine
8000
7000
6000
5000
4000
3000
2000
1000
anti-IgM - + + - + +
BAFF - - + - - +

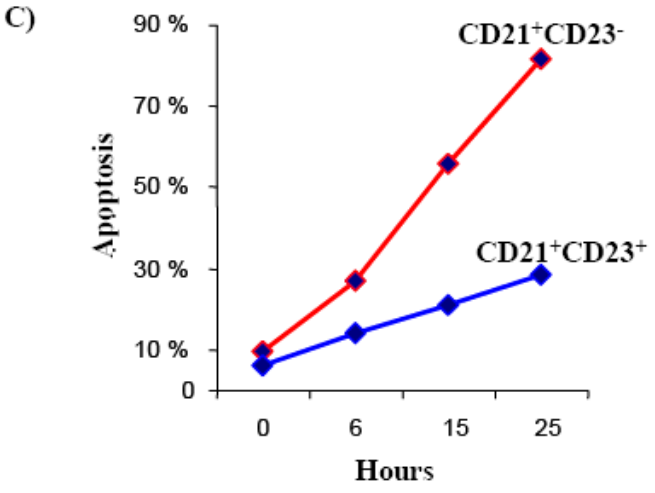

C)
90 %
70 %
50 %
30 %
10 %
0
Apoptosis
CD21+CD23-
CD21+CD23+
0
6
15
25
Hours

Figure 28: Functional analysis of $CD23^+$ and $CD23^-$B cells from appendix. A) Flow cytometric analysis of B cells for CD23, CD21 and CD24 B) Proliferation of FAC-sorted $CD21^+CD23^-$ and $CD21^+CD23^+$ B cells cultured for 3 days in the presence of anti-Ig (10µg/ml) and sBAFF (3 µg/ml). Cells were pulsed with [^{3}H]-thymidine during the last 18 hrs in culture. C) B cell subsets used in B) were assessed for their ability to undergo apoptosis upon BCR crosslinking. Percent apoptotic cells at each time point were calculated based on staining with annexin V and propidium iodide.

expressing several activation markers were maintained, presumably by self-renewal (Hao and Rajewsky, 2001). Because there is no influx of new B cells from the BM of adult rabbits, I tested if rabbit B cells exhibit an activated phenotype. I analyzed appendix and splenic IgM^+ B cells for the expression of surface CD44, CD25, CD80/86, CD11b, and CD43, markers that are characteristic of activated cells (Fig 29). I found that only a fraction of appendix B cells were positive or stained weakly for these markers. The low or weak expression of these activation markers in the appendix could be due to the presence of only a small number of $CD23^+$ mature B cells (Fig 29). In the spleen, however, I was surprised to find that essentially all B cells were positive for these markers, thereby exhibiting an activated phenotype. I was surprised because B cells in the murine spleen are largely resting and do not express detectable levels of surface CD80, CD86, CD69, and CD25 (Hsueh et al., 2002). As I discuss later, perhaps the activated phenotype on rabbits B cells could be due to a chronic stimulation of the BCR by tonic/survival signals that enable them to remain long-lived after the arrest of lymphopoiesis in the BM.

Phenotypic analysis of rabbit B cell lines

The Knight lab generated three rabbit B cell lines: 55D1, PBL-1 and 79E that were derived from the spleen, PB and MLN, respectively of *c-myc* transgenic rabbits (Knight et al., 1988, Sethupathi et al., 1994). To facilitate the use of these cell lines in investigating the biology of rabbit B cells, I characterized them based on the expression of activation and CD markers (Fig 30A and B). I found 79E B cells

($CD20^{+}CD21^{+}CD27^{+}CD38^{+}$ and $CD44^{-}$) were distinct from both 55D1 and PBL-1 ($CD20^{-}CD21^{-}CD27^{lo/-}CD38^{lo/-}$ and $CD44^{+}$) cells. The expression of CD27 on 79E B cells suggests to me that they could represent MZ or memory-like B cells. As for CD20 expression on 79E cells, I will discuss later that CD20, although, a marker for transitional B cells, can also be expressed at low levels on mature B cells. In conclusion, based on a $CD20^{+}CD21^{+}$ $CD27^{+}$ phenotype, I suggest that 79E B cells are mature B cells. It is difficult to draw a conclusion about the nature of 55D1 and PBL-1 B cells based on the phenotypes they present; $CD21^{-/lo}$ (Fig 30B), suggestive of a transitional B cell phenotype, and surface $CD24^{lo/-}$(data not shown), which is suggestive of a mature B cell phenotype. Functionally characterizing 55D1 and PBL-1 B cells will provide further insights into the nature of these B cell lines.

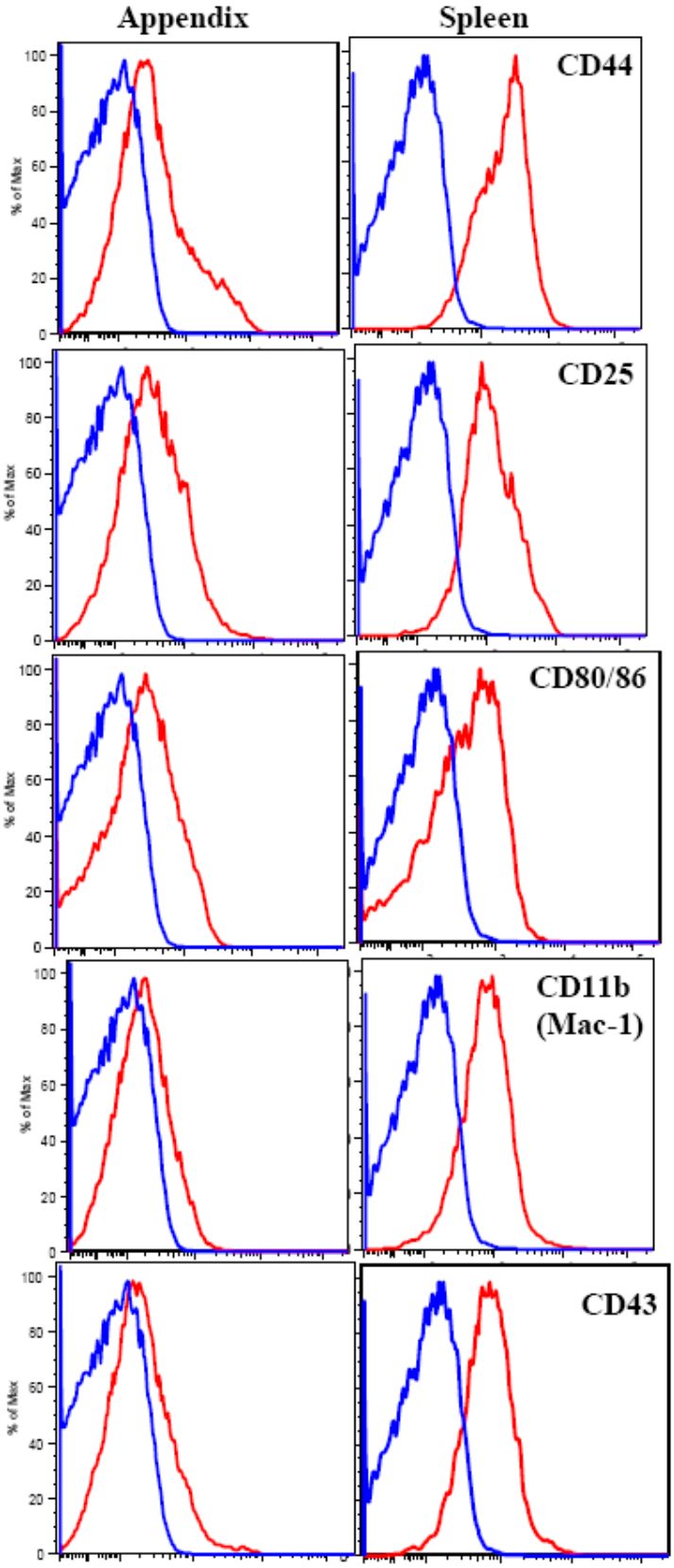
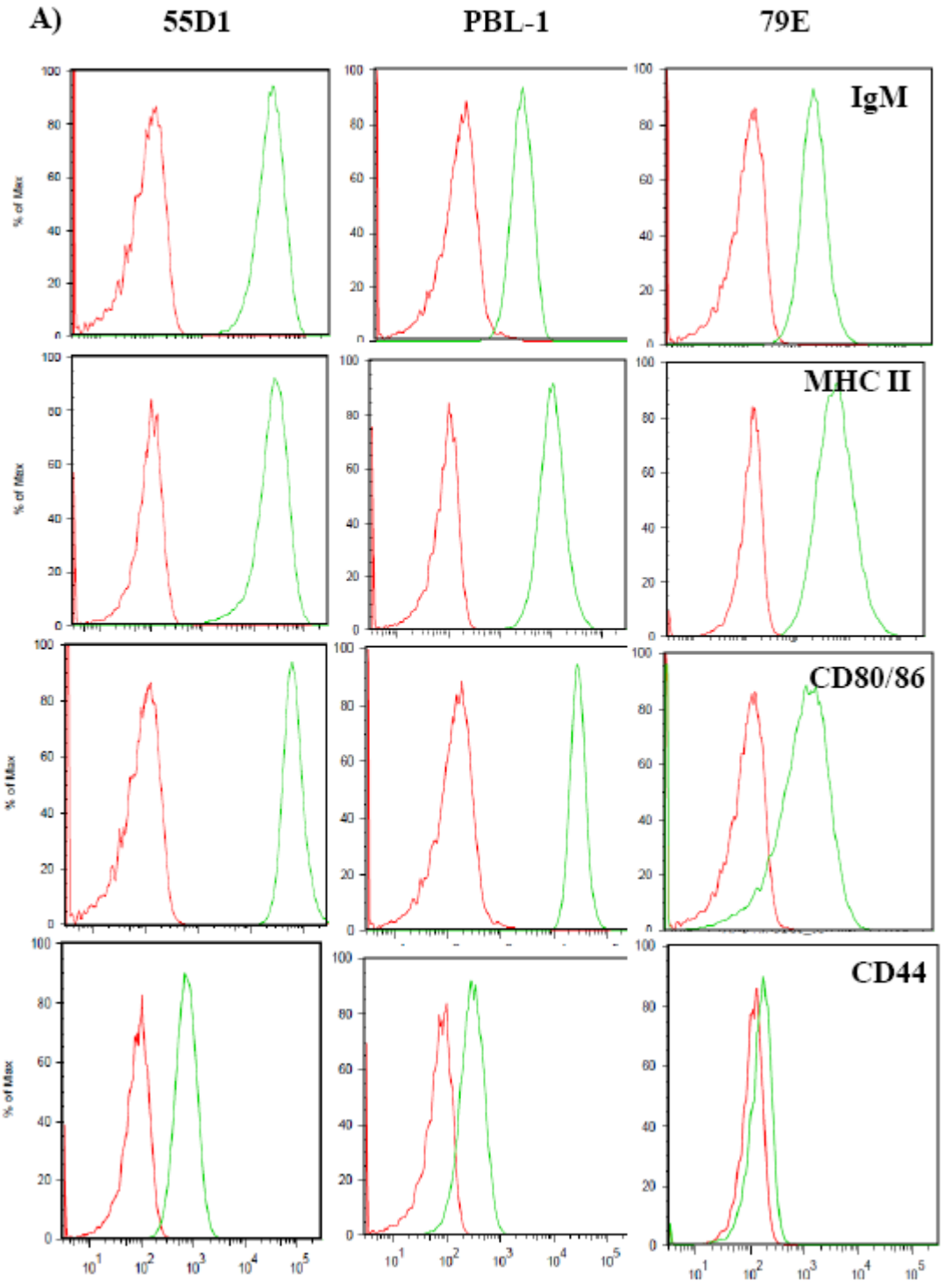

Appendix
Spleen
CD44
CD25
CD80/86
CD11b
(Mac-1)
CD43
% of Max
0
20
40
60
80
100

Figure 29: Expression of activation markers on splenic and appendix B cells.

Histograms (red) showing the expression of CD44, CD25, CD80/86, CD11b, and CD43 on IgM^+ (gated) B cells from the appendix and spleen. Blue histograms = isotype control.


A)
55D1
PBL-1
79E
IgM
MHC II
CD80/86
CD44
% of Max

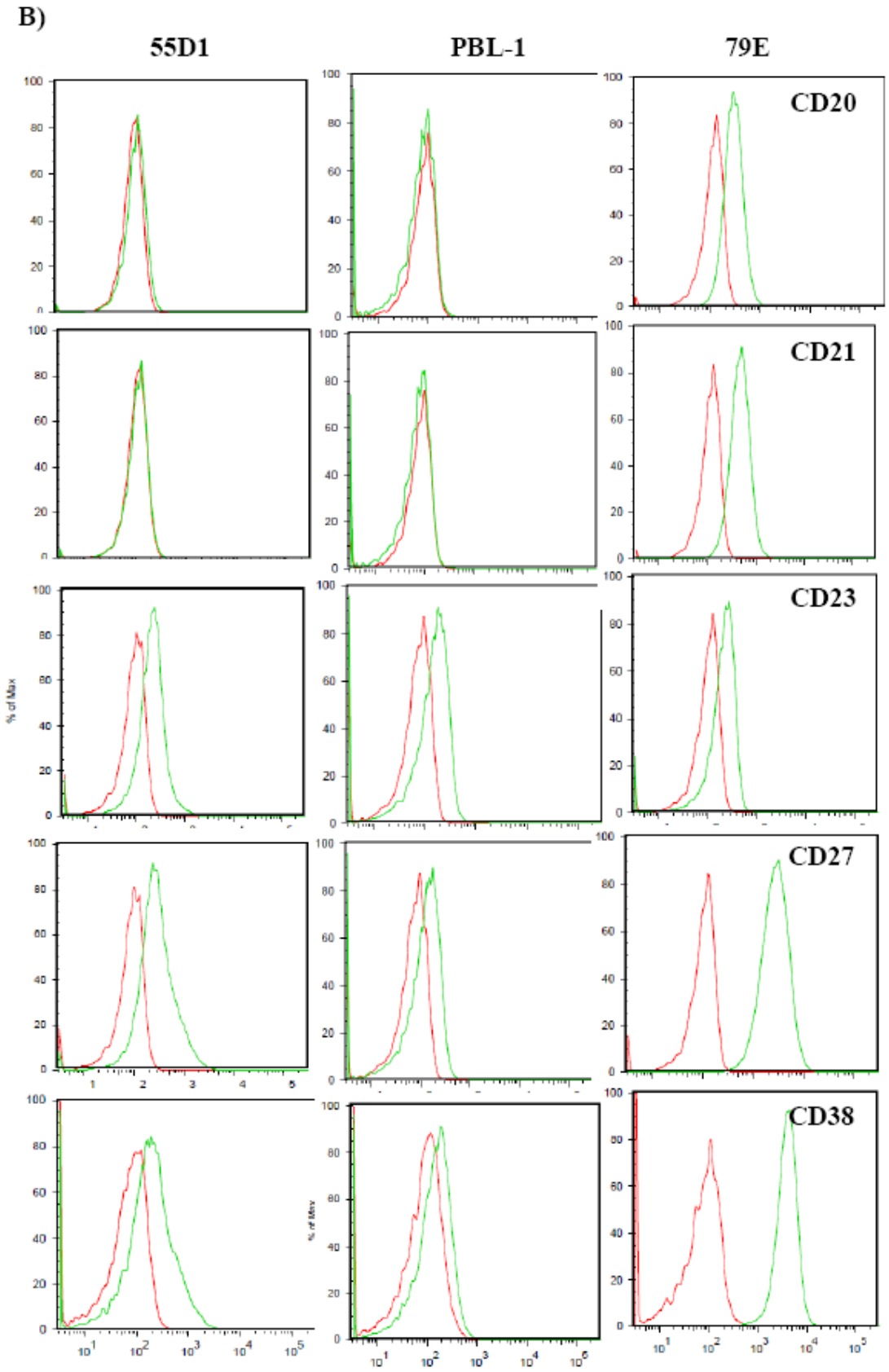

B)
55D1
PBL-1
79E
CD20
CD21
CD23
CD27
CD38
% of Max

Figure 30: Phenotypic analysis of rabbit B cell lines; 55D1, PBL-1 and 79E. A) Histograms (green) showing the expression of IgM, MHC II, CD80/86 and CD44. Red histograms = isotype control. B) Histograms (green) showing the expression of CD20, CD21, CD23, CD27 and CD38. Red histogram = isotype control.

CHAPTER FOUR

DISCUSSION

Mammals develop and maintain their B cell compartment through unique and conserved mechanisms. In the process of investigating how B cells develop in rabbits, I identified several immature and mature B cell subsets based on phenotypic and functional characterization (summarized in table 4). Compared to other species, several unique features of the B cell compartment were identified: (1) transitional B cells are present in the peripheral tissues of adult rabbits long after the arrest of lymphopoiesis; (2) transitional B cells in the periphery are proliferating and somatically diversified; (3) with the exception of transitional and splenic MZ B cells, the BAFF-binding receptors of essentially all B cells are occupied; (4) mature B cells in the periphery have an activated phenotype, and (5) splenic $CD27^{+}$ MZ B cells are absent in GALTless rabbits. While these findings provide insights into how B cells develop, and are maintained; they also raise several questions that need to be addressed to further understand rabbit B cell biology. In the following sections, I will discuss these and other findings and the implications they have for B cell development and homeostasis. I will also raise important questions to be addressed and end with a model of B cell development in rabbits.

Table 4: Phenotypes and distribution of B cell subsets in adult rabbits

Subset	**Phenotype**	**Tissue Distribution[a]**
T1[b]	$CD24^{hi}CD21^{lo}$	Apx, SR, PP, MLN, Spl, PB
T2	$CD24^{hi}CD21^{+}$	Spl
M[c]	$CD24^{lo/-}CD21^{+}$	Apx, SR, PP, MLN, Spl, PB, and BM
MZ[d]	$CD23^{-}CD9^{hi}CD1b^{+}$ $CD27^{+}$	Spl
GC	$PNA^{hi}CD23^{-}CD62L^{-}$ $BCL6^{+}$	Apx, SR

[a]Appendix (Apx); Sacculus rotundus (SR); Peyer's patch (PP); Mesenteric lymph node (MLN); Spleen (Spl); Peripheral blood (PB); Bone marrow (BM)

[b]T1 B cells are also $IgM^{lo}CD62^{lo}$ when compared to T2 and Mature (M) B cells that both express higher levels of IgM and CD62L

[c]Mature follicular B cells are also $CD23^{+}$

[d]$CD27^{+}$ MZ-like B cells are also found in Apdx, SR, PP, MLN, and PB

Transitional B cells

Identification and characterization. As I mentioned previously, one major difficulty in identifying B cell subpopulations in rabbits and other related species is the lack of antibody reagents. I overcame this difficulty by using cross-reactive antibodies and identified subsets of rabbit immature B cells using anti-CD24 mAb. The $CD24^{hi}$ transitional B cells were classified into two subsets: T1 and T2 ($CD21^{lo}$=T1; $CD21^{+}$=T2). Phenotypically, these transitional B cell subsets exhibited some differences and similarities from their counterparts in other species. Unlike in mice, where T1 B cells are $IgD^{-}IgM^{hi}$ and T2 B cells are $IgD^{hi}IgM^{hi}$ (Loder et al., 1999), transitional B cells in rabbits differ only in the relative expression of IgM (T1=IgM^{lo}; T2=IgM^{+}). There is no evidence for the expression of an IgD-like molecule on rabbit B cells (Knight and Winstead, 1997). Both T1 and T2 B cells were $CD23^{+}$, unlike in mouse where T1 B cells are $CD23^{-}$ and T2 B cells are $CD23^{+}$ (Loder et al., 1999). Based on the expression of CD23, CD10, CD38 and CD20, rabbit transitional B cells appear similar to human transitional B cell subsets. Like in rabbits, CD23 is expressed on both human T1 and T2-like subsets and thus is not a useful marker to distinguish these B cell subsets (Suryani et al., 2010). Further, as in humans, I found CD10 and CD38 could be used to characterize rabbit transitional B cells. In humans, CD20 is expressed at high levels on transitional B cells and also on all mature B cells (Sims et al., 2005), and thus serves as a pan B cell marker. Interestingly, in rabbits, I found CD20 expressed only on transitional B cells. I cannot however, rule out the possibility that CD20 is expressed at undetectably low levels

on mature B cells. Nevertheless, this reagent (anti-CD20 mAb; clone B9E9) served as a unique and additional marker for me to identify transitional B cells. In table 5, I summarize the phenotypes of rabbit transitional B cells and compare them with human and murine transitional B cells.

Functionally, transitional B cells in rabbits exhibit some similarities with their counterparts in other species. In mice, BAFF is required for development of transitional B cells as shown by the loss of mature B cells and the arrest of B cell maturation at the T1 B cell stage in BAFF-deficient animals (Gross et al., 2001). By neutralizing BAFF in neonatal rabbits, I demonstrated a similar loss of mature B cells and an arrest in B cell development at the T1 B cell stage, confirming that transitional B cells in rabbit also depend on BAFF for their development. Further, the absence of T2 and mature B cells in the spleen following BAFF neutralization suggests that, similar to mice, T1 B cells in rabbit are the earliest known precursor during peripheral B cell development and that these cells likely give rise to T2 and mature B cells. I therefore characterized only T1 B cells for subsequent functional analysis. Lastly, consistent with studies in mice and humans (Allman et al., 1992, Sims et al., 2005), T1 B cells in rabbit did not enter cell-cycle upon anti-Ig stimulation, while mature B cells readily entered cell-cycle under similar conditions.

In addition to the similarities between rabbit and mouse transitional B cells discussed above, T1 B cells in rabbit exhibit some unique characteristics. While murine T1 B cells are largely non-cycling *in vivo* (Loder et al., 1999, Allman et al., 2001), I was

Table 5: Phenotypes of transitional B cells in rabbit, human, and mouse

Marker	Rabbit[a]		Human[b]		Mouse[c]	
	T1	T2	T1	T2	T1	T2
CD24	+++	+++	+++	+++	+++	+++
CD21	+	++	+	++	-/+	++
IgM	+	++	+++	+++	+++	+++
CD62L	+	++	+	++	-	+
CD23	+	+	+	+	-	+
CD10	+	++	++	++	ND	ND
CD38	+	++	+++	++	ND	ND
CD20	++	++	+++	+++	ND	ND
CD90	+++	++	ND	ND	ND	ND

+++ = High ++ = Intermediate + = Low - = Negative

ND = Not determined (or not a marker)

[a] As characterized on splenic B cells

[b] (Carsetti et al., 2004,Sims et al., 2005, Marie-Cardine et al., 2008)

[c] (Loder et al., 1999, Allman et al., 2001, Allman and Pillai, 2008)

surprised to find that essentially all rabbit T1 B cells were proliferating *in vivo*. Further, unlike in mice and humans (Mao et al., 2004, Sims et al., 2005), rabbit T1 B cells have somatically diversified Ig genes. A diversified repertoire indicates that these cells have undergone a GC-like reaction and are not recent emigrants from the BM. Further, the presence of diversified T1 B cells in adult rabbits, long after the arrest of B lymphopoiesis, suggests that these B cells are maintained in the periphery, possibly because they are long-lived and/or self-renewing. Lastly, the rate at which transitional B cells mature in rabbit appears to be faster than in mouse. HSA^{hi} immature/transitional B cells constitute most of the peripheral B cells in neonatal mice. They reach adult levels in the spleen (5-10% of all B cells) only after 6-8 weeks of age (Allman et al., 1992, Allman et al., 1993). In contrast, in rabbits, $CD24^{hi}$ transitional B cells are already present at the levels seen in adults by approximately 7 days of age, suggesting that transitional B cells rapidly mature in the lymphoid tissues, after they leave the BM. Because B lymphopoiesis occurs only early in life, rabbits may have evolved this strategy to rapidly differentiate their immature B cells and generate a functional B cell compartment before the arrest of B lymphopoiesis.

Localization in GALT. Analysis of neonatal (3 and 6 day old) rabbits revealed that $CD20^{+}$ transitional B cells are localized in the domes and villous regions of GALT. These findings suggest that BM-derived immature B cells first migrate to the domes and villous regions of the appendix before differentiating into follicular B cells. Why do transitional B cells enter domes and villi? What could be the significance of this unique pattern of

localization? Because M cells in GALT are known to both translocate bacteria and sample luminal antigens (Miller et al., 2007), I suggest that transitional B cells in the domes that are in close proximity to the M cells interact directly with commensal bacteria or with bacterial-derived products. Such interactions in GALT may promote further differentiation and/or selection of B cells during peripheral B cell development. In support of this idea are the following observations made in rabbits and mice. Both remote colony and ligApdx rabbits, which have either limited and no microbiota, respectively, in the appendix, have reduced numbers of peripheral B cells, when compared to conventional rabbits (Lanning et al., 2000a). Similarly, the number of mature B cells in germ-free mice is strongly reduced, while the numbers of T2 B cells remain normal (Loder et al., 1999). Lastly, in a naturally occurring mutant rabbit, Rhee et al. (2005) demonstrated that during ontogeny, commensal bacteria are required for the positive selection of V_Ha allotype bearing B cells over V_Hn B cells. Taken together, these findings suggest that commensal bacteria play a role in inducing the differentiation and/or selection of B cells, presumably at the transitional B cell stage when they are present in the domes and villi of GALT.

B cell development in GALT

Experimental models of B cell depletion followed by reconstitution have proved useful in understanding the kinetics and stages of peripheral B cell development in other species (Allman et al., 1993, Marie-Cardine et al., 2008, Palanichamy et al., 2009). The Knight lab fortuitously developed such a model when they generated IgH transgenic

rabbits, which were B cell deficient at birth. The subsequent appearance of IgM^+ B cells in these animals, first in the appendix and SR, and later in PP, MLN, PB, and spleen suggests that during development, B cells first migrate to the appendix and SR before populating other tissues (Jasper et al., 2007). I identified that the IgM^+ B cells in the appendix of an IgH Tg rabbit were predominantly $CD20^+$ transitional B cells and were localized in the domes and villi, similar to the distribution of transitional B cells in conventional neonatal rabbits. Taken together, the appearance of IgM^+ B cells in the appendix and SR of IgH Tg rabbits, and their localization in the domes and villi suggests that during B cell development, (1) T1 B cells first migrate to the appendix and SR, and (2) enter the domes and villous regions, before maturing into follicular B cells.

Stages of B cell maturation. How do T1 B cells mature in GALT? Following the identification of T1 and T2 B cells in the murine spleen, Loder et al. (1999) proposed a linear T1→T2→M pathway for B cell maturation. They found the intermediate T2 B cells only in the spleen, while T1 B cells were found in blood, spleen, and BM. In rabbits, consistent with the established role of GALT during early B cell development, T1 B cells were also found in the appendix, SR, PP, and MLN, as well as in spleen and blood, while T2 B cells were found only in spleen. Based on these findings, I suggest that B cell maturation in the rabbit may also proceed in a murine-like T1→T2→M pathway. In GALT, although I did not find a phenotypically distinct T2-like B cell population, the presence of a small number of such intermediates cannot be ruled out. Alternatively, B cell maturation in rabbit GALT may be distinct and not involve a T2 B cell-like stage, but

may proceed directly in a T1→M pathway. In support of such a pathway, Roundy et al. (2010) recently demonstrated that MZ B cells can in fact be directly generated from immature/T1 B cells. Further, using mathematical models of the dynamics of peripheral B cell subsets, Shahaf et al. (2004) provided evidence for the existence of alternate or non-linear pathways for peripheral B cell maturation.

During the transitional stage of B cell development, a large number of self-reactive B cells that escape selection in the BM are purged from the mature B cell repertoire (Carsetti et al., 1995). In humans, approximately 40% of the transitional B cells are auto-reactive, and only about 50% of them develop into mature B cells (Wardemann et al., 2003, Meffre et al., 2004, Wardemann and Nussenzweig, 2007). Where are self-reactive transitional B cells eliminated? Transitional B cells are first found in GALT, and I suggest that it is in GALT that auto-reactive B cells are eliminated. An optimal level of BAFF is required for B cell development as evidenced by the lack of B cell development in the absence of BAFF (or low levels thereof), and the development of autoimmunity due to the escape of autoreactive B cells in the presence of excess BAFF (Gross et al., 2001, Mackay et al., 1999). BAFF expression in GALT is induced by commensal bacteria, and I suggest that commensals regulate and maintain optimal levels of BAFF, and thereby promote selection of transitional B cells. In support of this idea, rabbits with GALT removed at birth (neonatal GALTless) developed an autoimmune-like phenotype, whereas surgical removal of the spleen had no effect (Cooper et al., 1968). It appears that in the absence of GALT, auto-reactive B cells accumulate in the periphery and secrete

autoantibodies. This idea can be tested further by determining if the frequency of transitional B cells in the spleen and other lymphoid tissues of neonatal GALTless rabbits is increased.

Expansion of follicular B cells in GALT. Following the differentiation of transitional B cells in GALT, large GC-like structures are formed due to the proliferative expansion of follicular B cells. In the following paragraphs, I discuss some mechanisms by which B cell expansion in GALT occurs.

To address whether B cells develop in a T cell independent manner, I attempted to deplete T cells by surgical removal of the thymus and injection of anti-CD4 mAb at birth. I found that neither of these treatments was effective in depleting T cells, presumably because either T cells were already seeded in the periphery at birth and/or the level of anti-CD4 mAb was insufficient. These treatments would likely be more effective in depleting peripheral T cells if they are performed *in utero*. Since this option was not available, I took an alternate approach and inhibited T cell activation by interfering with B7:CD28 costimulation. The absence of a developmental phenotype in the appendix of CTLA4-Ig treated rabbits, suggested to me that B cells in rabbit GALT develop in a T cell independent manner. I found, however, that CD40:CD40L interactions are required for B cell proliferation in GALT and although, the source of CD40L would usually be activated T cells, several other cell-types, including DCs, macrophages, NK cells, basophils, eosinophils, epithelial and endothelial cells, and B cells, (Schonbeck and Libby, 2001) also express CD40L. I predict that these other cell types are the source of

CD40L. In support of this idea, I demonstrated that CD40L is expressed throughout the appendix tissue, not only in the T cell zone. To definitively demonstrate that T cells are not required for peripheral B cell development, I recommend additional experiments in which T cells are depleted by injection of anti-CD4 immunotoxin, e.g., anti-CD4 conjugated to ricin.

B cells in GALT could also be stimulated to proliferate through the engagement of complement receptor CD21 (CR2) with its ligands; iC3b, C3d, and C3dg, breakdown product of the complement pathway. CD21 on B cells is part of a co-receptor complex of proteins: CD19 and TAPA-1 (CD81), which are known to provide co-stimulatory signals (Matsumoto et al., 1993). The BCR and the co-receptor complex are crosslinked, when CD21 binds to complement-coated antigen that has been captured by surface Ig; this crosslinking results in the activation and proliferation of B cells (Carter et al., 1988, Dempsey et al., 1996). By interfering with the interaction of CD21 and its ligand(s), and also by depleting C3 with CVF, I showed that complement is required for B cell development in GALT. The C3 staining I found in the appendix follicles could be due to the deposition of complement-coated antigens on follicular B cells and/or on FDCs that also express complement receptors CD21 and CD35 (CR1). In GCs, FDCs are described as potent accessory cells for stimulating B cells. They trap complement-coated (opsonized) Ag-Ab complexes [Immune complexes (IC)], and interact with B cells by providing a FDC Ag- BCR interaction and a co-signal through B cell CD21-FDC CD21L (opsonized antigen) (Tew et al., 2001). In GALT, I propose that B cells could be

activated by FDCs that trap ICs made of bacterial-derived molecules and IgA. These ICs could be derived from commensal bacteria that are coated with C3 and IgA. Alternatively, the opsonized bacteria may be translocated by M cells and transported into the follicles where B cells directly interact with bacteria and complement. The continuous availability of such gut-derived microbial antigens in the follicles may support B cell proliferation and expansion in GALT.

Little is known about the role of complement during B cell development in GALT. Most studies about complement-mediated regulation of B cells are performed within the context of an infection or humoral immune response (Chen et al., 2000). Using GF appendices into which either no bacteria or select bacterial species were introduced, I demonstrated that commensal bacteria regulate the expression/deposition of C3 in the follicles. Further, because C3 was found in the presence of *B. fragilis* and *B. anthracis*, which are both known to promote GALT development, but not in the presence of *E. coli*, which does not induce GALT development, I suggest that one of the mechanisms by which select commensals induce GALT development is through the activation of the complement pathway. The bacteria could activate the classical pathway through IC formed by natural IgM and bacterial-derived antigens and in turn, the IC could stimulate B cells. Alternatively, bacteria could activate the mannose lectin-binding pathway which is independent of Ig binding to the bacteria. Consistent with this latter pathway Casola et al. (2004) showed that in mice that lack serum Ig, the complement cascade was activated in the PP GCs. The authors suggested that activation occurred through the alternative or

mannose-lectin binding pathway (Casola and Rajewsky, 2006). Further studies to understand how commensals promote complement activation, and thereby B cell developments in GALT need to be performed.

Mesenchymal stromal cells (SCs) could also stimulate GALT B cells to proliferate in a contact-dependent manner. Griebel and Ferrari (1994) isolated SCs from sheep ileal PP and generated a number of clones, and found that some of them supported proliferation of ileal PP B cells, but not B cells from blood. Further, they found that the proliferation was independent of antigen and T cells, but dependent on contact with SCs. Neither the mechanism, nor the molecules involved in these B cell-SC interactions is known. I predict that similar unique interactions may occur between B cells and SCs in the rabbit GALT, and such interactions may contribute to the proliferative expansion of B cells. This idea can be tested *in vitro* by co-cultures of appendix B cells and the sheep PP-derived SC cell lines. If appendix B cells are stimulated by these SC lines, then I predict that B cells would proliferate in these co-cultures. If sheep-derived SCs do not stimulate B cells, then rabbit SCs could be isolated and cloned from the appendix and tested for their ability to stimulate B cells.

Dramatic reduction in the size of proliferating B cell follicles in the appendix by administration of TACI-Ig indicated that BAFF/APRIL is required during the early phase of B cell development in GALT. In addition to promoting maturation of B cells, BAFF may also aid in the recruitment of transitional B cells from the BM to GALT. Badr et al. (2008) recently reported a novel role for human BAFF in B cell chemotaxis by showing

that BAFF increased the chemotactic response of primary human B cells to a few chemokines, including CXCL13. Interestingly abundant expression of BAFF and CXCL13 (Hanson and Lanning, 2008) is found in the developing rabbit appendix, suggesting that BAFF may similarly enhance the chemotactic response of incoming B cells to CXCL13 produced by resident stromal cells/ FDCs. Results from the *in vitro* analysis of appendix B cells, in which BAFF provides a survival rather than a proliferative signal to B cells, suggests that BAFF plays a role in survival of the expanded follicular B cells. Lastly, although TACI-Ig can bind and therefore neutralize both BAFF and APRIL, the phenotype observed in TACI-Ig treated rabbits appears similar to BAFF-deficient mice, but not APRIL-deficient mice (in which B cell development is normal), suggesting that the lack of BAFF rather than APRIL is likely responsible for inhibiting B cell development in GALT.

I have not investigated the role of TLRs in GALT development, but these may well have an important role in this development. The role of TLRs could be investigated by disrupting MyD88 signaling in neonatal B cells through overexpression of Tollip, a negative regulator of TLR signaling (Burns et al., 2000, Bulut et al., 2001, Zhang and Ghosh, 2002). Mailly et al. (2006) developed a modified adenoviral vector, by inserting a CD21 binding sequence, (derived from Epstein-Barr virus GP350/220 protein) into the Ad fiber protein. Consequently, the tropism of the virus is modified such that it recognizes and binds to $CD21^+$ B cells. If this modified Ad vector also binds to rabbit $CD21^+$ B cells, then it could be used to overexpress Tollip in neonatal B cells. If TLR

signaling is required for B cell development in GALT, then overexpressing Tollip in B cells will block TLR signaling, and consequently inhibit B cell proliferation and expansion. Additionally, by determining which TLRs are expressed on B cell subsets in the appendix, we can predict which TLR ligands promote B cell proliferation in GALT. Several anti-human TLR antibodies (TLR2, 3, 4, 6, 8, and 9) that cross-react with rabbit (Dr. Lbachir BenMohamed; personal communication to Dr. Knight) are available for these studies.

In summary, B cells in the appendix expand in a B7-CD28 independent, and CD40-CD40L and CD21-CD21L dependent manner. BAFF, in addition to promoting differentiation of transitional B cells, likely provides a survival signal for the maintenance of B cells in the follicles.

Characteristics of mature B cells in GALT. Phenotypic and functional analysis of B cells from various compartments of GALT revealed that the composition of B cells in the appendix and SR were distinct. These tissues had only a few $CD23^{+}$ mature B cells; instead, most B cells were $CD23^{-}$ PNA^{hi} GC B cells. In contrast, PP was similar to spleen with mostly $PNA^{lo}CD23^{+}$ mature B cells. These findings are reminiscent of ileal and jejunal PP in sheep, where the former is a primary lymphoid organ, containing mostly $BAQ44^{-}$ immature/GC B cells, while the latter is thought to function as a secondary lymphoid organ, due to the presence of a predominant $BAQ44^{+}$ mature B cell population (Yasuda et al., 2006). Similarly, in rabbits, the appendix and SR may serve as primary lymphoid organs, while the PP may function as a secondary lymphoid organ. This

proposal is supported by the observations in IgH Tg rabbits, that IgM^+ B cells are first detected in the appendix and SR, prior to their detection in other GALT compartments- PP and MLN. Further, unlike ileal PP in sheep, the appendix and SR in rabbit do not involute with age, suggesting that these tissues may continue to function as primary lymphoid organs even in adults. A few GC-derived $CD23^+$ mature B cells from the appendix and SR may continuously seed the periphery and maintain peripheral B cell homeostasis, when there is no influx of B cells from the adult BM. Taken together, the characterization of B cells from different compartments of GALT, revealed a previously unappreciated aspect of appendix and SR B cells. During B cell development, the transitional B cells that seed different compartments of GALT likely differentiate into distinct effector B cell subsets- GC B cells in appendix and SR, and mature B cells in PP.

What could be the function of the GC B cells that occupy most of the follicles in the appendix and SR? Because, they are $CD62L^-$, I do not think that these cells enter the periphery. Instead, as discussed above, I suggest that only the few $CD23^+$ B cells in the appendix and SR seed the periphery. Recently, Booth et al. (2009) found that PP B cells in sheep spontaneously secrete high levels of IL10, an immunoregulatory cytokine, and proposed that these IL10-secreting PP B cells are regulatory B cells (B_{regs}). They found that PP B cells did not respond to CpG stimulation, despite expressing high levels of TLR9, while B cells from other tissues readily responded to CpG stimulation. However, in the presence of an IL10 neutralizing Ab, PP B cells responded, and secreted CpG-induced IFN and IL12 (Booth et al., 2009). In rabbits, B cells from the appendix did not

proliferate in response to TLR7/8 agonists, while splenocytes and MLN cells exhibited a dose-dependent proliferative response (Pi-Chen Yam & Katherine Knight, unpublished observations). Given the similarities between sheep and rabbit GALT, I speculate that the unresponsiveness of appendix B cells could be due to the presence of IL10 expressing B_{reg}-like cells in the appendix. Chronic proliferation and diversification of Ig genes in the appendix may result in the generation of B cells with auto-reactive BCRs. I envision that the B_{reg}-like cells in these tissues could suppress the expansion of auto-reactive B cells and thus help prevent autoimmunity. In conclusion, I suggest that the appendix and SR play a role in maintaining peripheral B cell homeostasis, while PP and other GALT serve as secondary lymphoid organs and confer protection against microbial pathogens.

Identification of MZ B cells

MZ B cells, defined by their distinct localization in the MZ constitute a heterogeneous population of B cells with distinct surface markers. In both rodents and humans, they are broadly identified by the lack of CD23, which stains only B cells located in the follicular zone. In mice, MZ B cells are $CD9^{hi}$ $CD1d^{+}$ IgM^{hi} $CD38^{hi}$ and $CD21^{hi}$ (Oliver et al., 1997, Amano et al., 1998, Won and Kearney, 2002). In humans, MZ B cells are $CD1c^{+}$ IgM^{hi} $CD21^{+}$ and also $CD27^{+}$ (Weller et al., 2004, Ettinger et al., 2007, Sanz et al., 2008, Weill et al., 2009). Using these markers, I found that MZ B cells in rabbit exhibit some characteristics of both human and murine MZ B cells. Anti-CD23 mAb did not stain B cells in the margins of splenic B cell follicles, indicating that MZ B cells are $CD23^{-}$. A few B cells in the MZ expressed the MHC class-I-like molecule,

CD1b, similar to the expression of CD1 isoforms on human (CD1c) and murine (CD1d) MZ B cells. Because rabbits express several CD1 isoforms that are homologous to human CD1 isoforms (Hayes and Knight, 2001), it may be that rabbit CD1c is also expressed on MZ B cells, but I did not have access to a CD1c antibody to test this. Further, I found B cells in the MZ were $CD9^{hi}$, a phenotype shared with murine MZ B cells (Won and Kearney, 2002). Lastly, similar to humans, I found that CD27 was expressed on MZ B cells, and the $CD27^{+}$ B cells were IgM^{hi}, $CD21^{hi}$ and $CD38^{hi}$ compared to $CD27^{-}$ B cells.

Human MZ B cells are distinct from their counterpart in the mouse in three aspects. First, they are circulating, unlike murine MZ B cells, which are restricted to the spleen. Second, they constitute 30-40% of the B cells, unlike in mouse where MZ B cells represent only about 5-10% of the B cells. Lastly, human MZ B cells are somatically diversified, while in rodents, they are undiversified (Weill et al., 2009). I found that in rabbits MZ B cells appeared similar to human MZ B cells in all three aspects listed above. In the spleen, $CD27^{+}$ MZ B cells constituted 30-40% of the B cells and they were also present in GALT and PB, suggesting that they are circulating. Splenic MZ B cells (as identified by a $CD23^{-}IgM^{hi}CD9^{hi}$ phenotype) were also somatically diversified. Morphologically, $CD27^{+}$ B cells were larger in size than $CD27^{-}$ B cells and *in vitro*, like human MZ B cells, they secreted more Ig, and readily entered cell cycle upon stimulation with anti-Ig, compared to $CD27^{-}$ B cells. Based on these findings, I suggest that the peripheral B cell compartment in rabbits is similar to that in humans, where the $CD27^{+}$

and $CD27^-$ B cell subsets can be considered as equivalents of the murine MZ and conventional B2 B cells, respectively.

CD27 in humans is also a marker for memory B cells. Because $CD27^+$ MZ B cells are somatically diversified, some investigators define these cells as IgM memory B cells (Tangye and Good, 2007). CD27 is also expressed on bonafide class-switched memory B cells, which are known to reside in the MZ (Dunn-Walters et al., 1995, Klein et al., 1998). Based on these criteria, $CD27^+$ and $CD27^-$ B cells are broadly classified as memory and naïve B cells, respectively. In a preliminary experiment to test if CD27 is a marker for memory B cells in rabbits, I immunized an adult rabbit and FAC-sorted $CD27^+$ and $CD27^-$ B cells and stimulated them *in vitro* with CD40L and IL-4. By ELISA, I found antigen-specific antibodies (IgG) in the culture supernatants of $CD27^+$ B cells, but not $CD27^-$ B cells. Further, I found that CD27 was expressed on some class-switched (IgG and IgA) B cells in the spleen. These preliminary results suggest that CD27 may identify memory B cells as well as MZ B cells in rabbit. Further experiments to confirm these findings and test if CD27 is a marker for memory B cells need to be performed.

The absence of splenic $CD27^+$ MZ B cells in rabbits that had GALT surgically removed at birth indicates that the development of these mature B cell subsets is dependent on GALT. Three possible mechanisms by which MZ B cells can develop are: 1) Transitional B cells in GALT are, or become committed to a MZ fate and migrate to the spleen and differentiate in the MZ, 2) MZ B cells develop in GALT, and migrate to the splenic MZ, or 3) MZ B cells develop in spleen, and GALT is required for their

maintenance. Using notch2 and delta-like 1 (DL1) knockout mice, Saito et al. (2003) and Hozumi et al. (2004) showed that signaling through notch2 is required for MZ B cell development. It is proposed that T1 B cells come in contact with DL-1^{+} endothelial cells lining the marginal sinus of the spleen and differentiate into MZ B cells (Pillai and Cariappa, 2009). To test the possibility that transitional B cells in rabbit are committed to a MZ fate and develop into MZ B cells in GALT, I examined whether notch2 and its ligands are expressed in the appendix. The expression of the notch ligand, Jagged-1 near the FAE in the domes suggests to me that T1 B cells that enter these sites during ontogeny may come in contact with Jagged-1 or other notch2 ligand-expressing cells and either become committed to a MZ fate or differentiate into MZ B cells. In this regard, it will be important to determine if DL1 and other notch2 ligands are also expressed in GALT. If DL1 is expressed in GALT, then its ability to promote differentiation of rabbit transitional B cells into MZ B cells can be examined *in vitro*. Primary $CD24^{hi}$ T1 B cells from GALT could be co-cultured with OP9-DL1 cells plus BAFF, and the cultures examined for the development of $CD27^{+}$ $CD23^{-}$ mature MZ-like B cells. This co-culture approach was recently used by Roundy et al. (2010) to demonstrate that maturation of transitional B cells can be induced *in vitro*, and that the commitment to a MZ or follicular B cell fate can be dictated by DL1 and BAFF signals, respectively.

The origin and development of human MZ B cells ($IgM^{+}IgD^{+}CD27^{+}$) is controversial. Fully functional MZ B cells, as defined by the ability to mount an immune response to T cell independent antigens, are not formed until a few years after birth

(Timens et al., 1989). However, because $IgM^+IgD^+CD27^+$ MZ B cells with mutated Ig genes are already found in children below 2 years of age, it has been proposed that these cells develop in GALT, in a manner seen in species such as sheep and rabbits (Weller et al., 2004, Weller et al., 2008, Weill et al., 2009). The absence of splenic $CD27^+$ human-like MZ B cells in GALTless rabbits supports this possibility. Because of the similarities between human and rabbit MZ B cells, I propose that rabbits can be used as a model to understand human MZ B development and maintenance.

Maintenance of the peripheral B cell compartment in adult rabbits

The peripheral B cell compartment in many species is maintained by the continuous production of new B cells in the BM. In rabbits, however, B lymphopoiesis in the BM arrests by 4 months of age, and it is unclear how the peripheral B cell compartment is maintained when there is no influx of new B cells. In the following sections, I will discuss three unique features of the rabbit B cell compartment: 1) presence of proliferating and somatically diversified T1 B cells in adults, 2) occupied BBRs on B cells, and 3) activated mature B cells, and propose mechanisms by which the B cell compartment is maintained in adult rabbits.

Presence of T1d B cells in adult rabbits. How are transitional B cells maintained in adult rabbits and what is their significance? Because T1 B cells in adults are somatically diversified, I designate them as T1d B cells to distinguish them from undiversified immature B cells that are generated in the BM early in ontogeny. The Knight lab previously demonstrated that GALT is required for somatic diversification of Ig genes

(Vajdy et al., 1998) and that the Ig genes of essentially all peripheral B cells are diversified by 6-8 weeks of age (Crane et al., 1996). I suggest that T1d B cells are also likely generated by this time period in GALT and arise from the undiversified BM immigrants. Following the arrest of B lymphopoiesis in adults, the proliferating T1d B cells in GALT likely self-renew and thereby are maintained. Additionally, similar to self-renewing murine B1 B cells, T1d B cells may possess a restricted BCR specificity to certain self- and/or bacterial-antigens that promote their maintenance (Hayakawa et al., 1984, Pennell et al., 1984, Pennell et al., 1989a, Pennell et al., 1989b, Hardy et al., 1989). This idea of possessing a restricted repertoire can be tested by CDR3 spectratype analysis of T1d B cells.

If the adult rabbit BM does not generate new B cells after 2 to 4 months of age, then how is the peripheral B cell compartment maintained? I propose that it is maintained in GALT by self-renewing T1d B cells. By administering TACI-Ig into neonatal rabbits, I demonstrated that BAFF promotes the differentiation of T1 B cells into mature B cells. Similarly, in adult rabbits, T1d B cells presumably continue to develop into mature B cells in a BAFF-dependent manner, and thereby maintain B cell homeostasis. The few $CD23^{+}$ mature B cells that reside in the appendix and SR could likely be derived from T1d B cells. I propose that the $CD23^{+}$ B cells in appendix and SR continuously seed the peripheral tissues and maintain peripheral B cell homeostasis. *In vivo* labeling of lymphocytes in the appendix by injecting BrdU into the appendix artery, followed by analysis of the kinetics of appearance, and frequency of $BrdU^{+}CD23^{+}$ B cells in the

peripheral tissues could provide insight into the contribution of appendix-derived B cells in maintaining B cell homeostasis.

Although I think T1d-derived (progeny) B cells in GALT and other tissues likely maintain the peripheral B compartment, I cannot rule out the possibility that the BM may periodically generate a small number of new T1 B cells. Nagaoka et al. (2000) demonstrated that during an immune response to infectious agents, B lymphopoiesis in the murine BM is perturbed, and there is a temporary accumulation of immature B cells in the spleen. Similarly, it may be that B lymphopoiesis is temporarily reinitiated at low levels in adult rabbits following an immune response, and the new B cells generated during such a response could give rise to additional T1d B cells in GALT.

Occupied BBRs on peripheral B cells. Almost all human and murine B cells bind recombinant BAFF and express BR3 as the dominant receptor (Schneider et al., 1999, Thompson et al., 2001, Avery et al., 2003, He et al., 2004). However, in rabbits, I was surprised to find that most B cells from GALT and PB did not bind rBAFF. I showed that this lack of binding is not due to the absence of BR3, since almost all B cells expressed BR3. Rather, I found that this lack of binding is due to prior engagement of BBRs with endogenous BAFF. Carter et al. (2005) first demonstrated this phenomenon of BBR occupancy on human B cells isolated from patients with SLE who have 2 to 10 fold elevated levels of serum BAFF. They found that the level of labeled recombinant BAFF bound to freshly isolated B cells was significantly reduced in SLE patients compared to healthy controls due to occupied receptors. The saturated levels of occupied BBRs on

rabbit B cells raise the possibility that BAFF is present at high levels in the serum. I attempted to measure serum BAFF using the polyclonal anti-human BAFF reagent, and was unable to detect BAFF by ELISA, presumably due to low sensitivity of the assay. In the future, it will be important to measure serum BAFF using a recently described cross-reactive anti-human BAFF mAb (Kikly et al., 2009).

The presence of occupied BBRs on rabbit B cells could also be due to the rapid shedding of membrane BAFF on B cells. This possibility is supported by the weak staining obtained for membrane BAFF using TACI-Ig as a reagent. Little is known about the cleavage of membrane BAFF and how this process is initiated. Li et al. (2008) showed that Fcγ receptor cross-linking triggers a rapid release of soluble BAFF from myeloid cells. How is the cleavage of BAFF triggered on B cells? Most rabbit appendix B cells express Fcμα receptor (FcμαR) and have IgA bound to their surface (Kari Severson and Katherine Knight; unpublished observations). I hypothesize that binding of IgA to FcμαR in the mucosal tissues trigger the release of BAFF from the surface of B cells. To generate a tool for investigating the cleavage of BAFF on B cells, I developed a BAFF-GFP fusion construct, whereby the GFP tag is on the extracellular portion of BAFF. The role of IgA- FcμαR interactions in the cleavage of BAFF can be investigated by the analysis of cleaved BAFF-GFP proteins in the culture supernatants of cells co-transfected with BAFF-GFP and FcμαR constructs, and cultured either in the presence or absence of IgA. If binding of IgA to FcμαR triggers the release of BAFF, then one can

expect to find more BAFF-GFP protein in the culture supernatant of cells with IgA compared to those without IgA.

What could be the significance of occupied BBRs? I propose that the occupancy of BBRs on B cells provides a tonic/survival signal, and consequently enables B cells to remain long-lived. Because rabbit B cells themselves express BAFF, they could be stimulated by BAFF in an autocrine fashion, and thereby remain long-lived. Although constantly occupied BBRs may provide a positive signal, they can also negatively interfere with any ongoing peripheral B cell selection, as seen in BAFF-transgenic mice (Mackay et al., 1999). These observations suggest that the BAFF signaling in rabbit B cells with occupied BBRs must be carefully regulated to maintain B cell homeostasis. How could BAFF signaling be regulated? Recently, a novel isoform of BAFF, known as delta-BAFF, was shown to dampen the co-stimulatory functions of BAFF *in vitro,* and also interfere with the binding of sBAFF to BBRs (Gavin et al., 2003). *In vivo*, delta-BAFF transgenic mice had reduced numbers of B cells, in contrast to BAFF transgenic mice that had an increased number of B cells (Gavin et al., 2005). These findings indicate that the presence of delta-BAFF can modulate the response of B cells to BAFF. Thus, investigating if rabbit B cells also express a delta-BAFF-like isoform could provide insights into how BAFF signaling is regulated in B cells with occupied BBRs.

Activated phenotype of mature B cells. The expression of activation markers on essentially all splenic and mature B cells in the appendix suggests that B cells in adults are activated. This is in contrast to the mouse, where essentially all the conventional B2

B cells in the spleen are in a resting state and do not express detectable levels of activation markers such as, CD69, CD25, CD80, and CD86 (Hsueh et al., 2002). However, in mice, in which B cell development in the BM was blocked, the few B cells (mostly MZ and B1 B cells) that were maintained in the periphery had an activated phenotype (Hao and Rajewsky, 2001). An activated state of these B cell subsets could presumably be due to chronic engagement of the BCR with tonic/survival signals and/or constant recognition of self-antigens that may promote maintenance. Because rabbit B cells also appear to be in an activated state, they may utilize some of the strategies adopted by MZ and B1 B cells for their maintenance. These cells could be self-renewing and/or long-lived. CD5, which is expressed on almost all rabbit B cells (Raman and Knight, 1992), has been shown to interact with surface Ig on rabbit B cells in a superantigen-like manner, and these interactions have been proposed to contribute towards the maintenance/longevity of B cells in adults (Pospisil et al., 1996). The chronic engagement of CD5 and its ligand(s) could be one reason why B cells are in an activated state. To investigate if rabbits B cells are long-lived and/or self-renewing, a rabbitized-mouse model can be developed by injecting T1d or T1 B cells into NOD/SCID mice. If these transitional B cells differentiate into mature B cells in the NOD/SCID mice, then the life-span and turn-over kinetics of rabbit B cells can be investigated in this model.

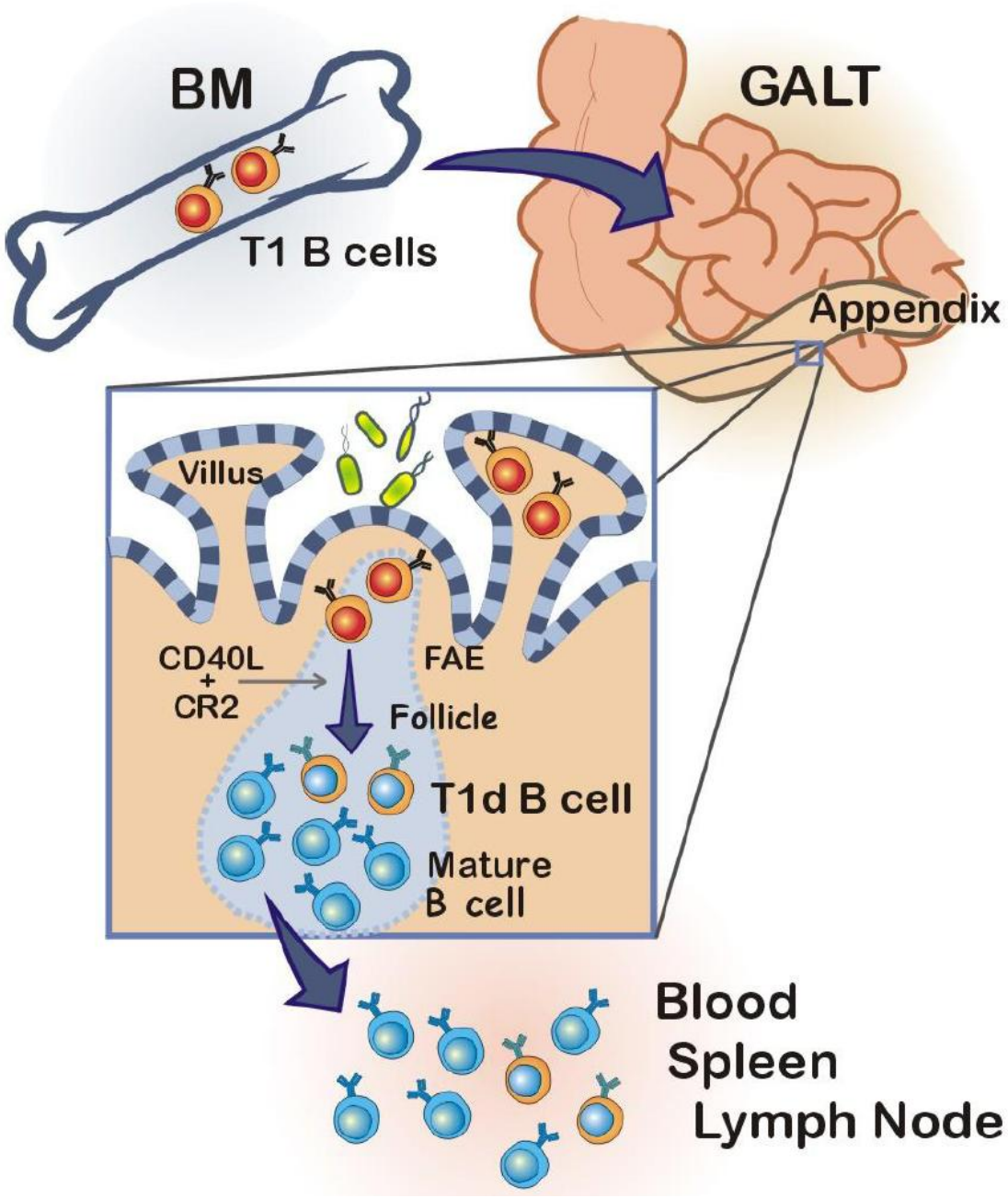
Model of peripheral B cell development

Based on my findings and previous observations made in the Knight lab, I propose a model (Fig 31A) of peripheral B cell development: $CD24^{hi}$ immature B cells

exit the BM early in ontogeny as transitional (T1) B cells ($IgM^{lo}CD21^{lo}CD62^{lo}$). These T1 B cells traffic to GALT (appendix) and enter the domes and villi (Fig31A *inset*). Here, they interact directly with either commensal bacteria or bacterial-derived products, such as superantigens (Severson et al., 2010) and become activated. Following activation, T1 B cells, proliferate, somatically diversify the Ig genes and differentiate into both follicular and T1d B cells. While the follicular B cells expand in a B7-CD28 independent, CD40-CD40L, and CR2-CR2L dependent manner in GC-like structures, the diversified T1d B cells leave the appendix and seed other peripheral tissues where they further develop to give rise to mature B cells. Alternatively, some of the BM-derived T1 B cells may also directly traffic to other sites, such as the spleen and develop into mature B cells. In the absence of newly-formed B cells from the BM, the T1d B cells that are presumably maintained in adult rabbits through self-renewal continually develop into mature B cells and thus maintain peripheral B cell homeostasis. Additionally, B cell homeostasis is maintained by long-lived mature B cells that have occupied BAFF-receptors (Fig 31B).

If my model of peripheral B cell development and maintenance in adult rabbits is correct, then I can make several predictions. First, if GALT maintains the peripheral B cell compartment by serving as a reservoir for T1d B cells, then following the surgical excision of organized GALT (GALTless) in adults, the frequency of peripheral B cells will decline over time. Further, if transitional B cells are not replenished by the BM and peripheral B cell homeostasis is maintained by T1d cells, then selective depletion of T1d B cells in adults would be expected to result in B cell lymphopenia. If GALT continues

A)


BM
T1 B cells
GALT
Appendix
Villus
CD40L
+
CR2
FAE
Follicle
T1d B cell
Mature
B cell
Blood
Spleen
Lymph Node

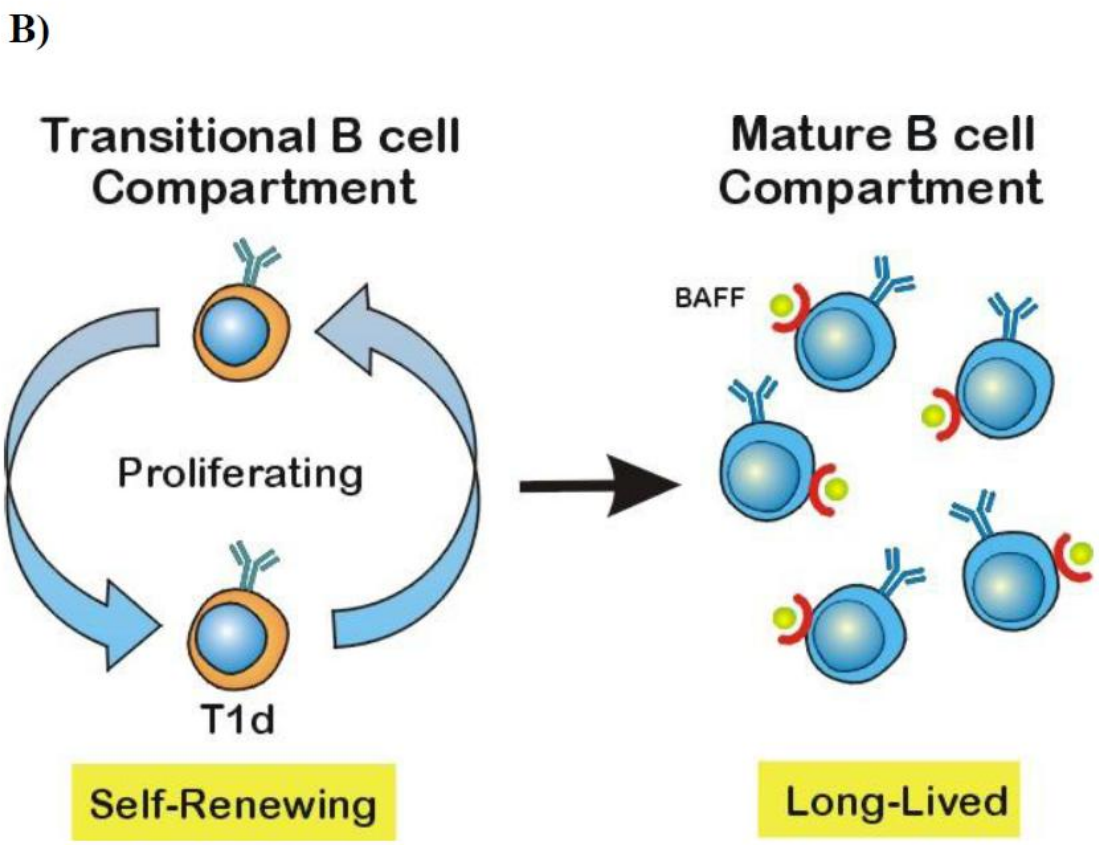


Figure 31: Model of peripheral B cell development and maintenance A) *Development of T1d B cells***.** T1 B cells leave the BM and enter GALT (appendix) through the HEVs and traffic to the domes and villi, where they are stimulated by BAFF and commensal bacteria or bacterial-derived products. The activated T1 B cells somatically diversify the Ig genes to become T1d B cells, which then directly differentiate into follicular B cells that undergo a proliferative expansion to form organized follicles in a CR2-CR2L and CD40-CD40L dependent manner. After undergoing the GALT GC-like reaction, mature and T1d B cells enter the circulation. Some of the GALT-derived T1d B cells traffic to the spleen and differentiate into T2 and

mature B cell subsets. Alternatively, some T1 B cells from the BM may directly traffic to the spleen and develop into mature B cells (not shown). B) *Maintenance of peripheral B cells***.** In adult rabbits, in the absence of ongoing lymphopoiesis, the B cell compartment is maintained by proliferating T1d B cells which self-renew and continually differentiate into mature B cells. Additionally, the BAFF receptor(s) on mature B cells in the periphery are bound by endogenous BAFF and this chronic engagement of BAFF receptors may provide a tonic/survival signal for the B cells to remain long-lived.

to function as a primary lymphoid organ in adult rabbits, then following depletion of mature B cells (but not T1d B cells) by a BAFF neutralizing agent [such as LY2127399, a crossreactive mAb (Kikly et al., 2009)] the rate of peripheral B cell reconstitution (after withdrawal of the BAFF neutralizing agent) will be much slower in GALTless rabbits compared to control B cell-depleted rabbits with intact-GALT. Lastly, another prediction is that both the BAFF-bound mature B cells and proliferating transitional B cells are long-lived and self-renewing, respectively. Experiments to test these ideas will elucidate some of the mechanism(s) by which rabbits and other species, that do not exhibit continuous B lymphopoiesis, develop and maintain their B cell compartment.

Concluding remarks

Different species have evolved different strategies to develop and maintain their B cell compartment. For my dissertation work, I focused on understanding how B cells in rabbits develop, and are maintained in adults. I identified and characterized transitional B cells in rabbits and by extension, in mammals that use GALT to develop their B cell repertoire. Remarkably, transitional B cells are maintained in the periphery of adult rabbits, when there is no evidence for ongoing lymphopoiesis in the BM. The finding(s) that these cells have a diversified repertoire and are undergoing proliferation confirms that these cells are not newly-made, and instead, leads to the idea that the T1d B cells are maintained by self-renewal and are responsible for maintaining the B cell compartment in the absence of detectable B lymphopoiesis. Further, the finding that B cells have occupied BBRs, suggests that rabbit B cells may be long-lived. These results provide

insights into how B cells develop, and are maintained not only in rabbits, but also in other species that exhibit limited B lymphopoiesis.

In 1979, Stewart Sell stated that most of our knowledge of the mammalian immune system is derived from studies in the mouse (Sell, 1979). This statement holds true even today, after almost three decades. In this article, the author discussed the advantages and disadvantages of using mice as a model to understand B cell biology. Although B cell development in mice appears similar to humans in some aspects, there are also significant differences, especially with regard to the composition of B cell subsets in the peripheral tissues. The presence of $CD27^+$ and $CD27^-$ B cells in rabbits suggests that rabbits have human-like memory and naïve B cell compartments. Further, many of the markers expressed on rabbit transitional B cells are also found on human transitional B cells, suggesting that rabbits can be used as a model to study human peripheral B cell development. Finally, I suggest that similar to rabbits, subsets of human transitional B cells may be found in the intestine and GALT and contribute to the maintenance of peripheral B cells as B lymphopoiesis in the BM decreases in the elderly.

In conclusion, as stated in chapter one, I would like to reiterate that the immune system of several species need to be investigated to obtain a holistic understanding of the immune system. It is my hope that the identification of several crossreactive antibody reagents and the functional characterization of rabbit B cells as described in this dissertation will serve as a basis for further studies in the rabbit and help establish rabbits as an alternate model to understand the human immune system.

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VITA

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The final copies have been examined by the advisor of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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